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(54) Title: METHODS FOR TREATING CANCER BY ADMINISTERING TUMOR-TARGETTED BACTERIA AND AN IMMUNOMODULATORY AGENT

(57) Abstract: The present invention provides combination therapies for the treatment of a solid tumor cancer in a subject. In particular, the present invention provides methods for treating a solid tumor cancer in a subject comprising administering to said subject one or more immunomodulatory agents and tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria. The present invention also provides methods for treating a solid tumor cancer in a subject comprising administering to said subject one or more immunomodulatory agents and tumor-targeted bacteria as vectors for the delivery of one or more therapeutic molecules. The methods of the invention improve the efficacy of tumor-targeted bacteria in the treatment of solid tumor cancers, while avoiding or limiting the adverse or unwanted side effects associated with the administration of bacteria to subjects whose immune system has been altered or compromised in some aspect by an immunomodulatory agent. The methods of the invention also provide more efficient local delivery of therapeutic molecules to the site of a solid tumor cancer using tumor-targeted bacteria engineered to express said therapeutic molecules. The present invention also provides compositions and kits comprising one or more immunomodulatory agents and one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria. The present invention further provides compositions and kits comprising one or more immunomodulatory agents and one or more tumor-targeted bacteria, wherein the tumor-targeted bacteria express one or more therapeutic molecules.

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METHODS FOR TREATING CANCER BY ADMINISTERING TUMOR-TARGETED BACTERIA AND AN IMMUNOMODULATORY AGENT

This application claims priority to U.S. provisional patent application No. 60/352,259, filed on January 28, 2002, which is incorporated herein by reference in its entirety.

1. FIELD OF THE INVENTION

The present invention relates to combination therapies for the treatment of a solid tumor cancer in a subject. In particular, the present invention relates to methods for treating a solid tumor cancer in a subject comprising administering to said subject one or more immunomodulatory agents and tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria. The present invention also relates to methods for treating a solid tumor cancer in a subject comprising administering to said subject one or more immunomodulatory agents and tumor-targeted bacteria as vectors for the delivery of one or more therapeutic molecules. The methods of the invention improve the efficacy of tumor-targeted bacteria in the treatment of solid tumor cancers, while avoiding or limiting the adverse or unwanted side effects associated with the administration of bacteria to subjects whose immune system has been altered or compromised in some aspect by an immunomodulatory agent. The methods of the invention also provide more efficient local delivery of therapeutic molecules to the site of a solid tumor cancer using tumor-targeted bacteria engineered to express said therapeutic molecules. The present invention also provides compositions and kits comprising one or more immunomodulatory agents and one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria.

2. BACKGROUND OF THE INVENTION

A neoplasm, or tumor, is a neoplastic mass resulting from abnormal cell growth, which can be benign or malignant. Benign tumors generally remain localized. Malignant tumors generally have the potential to invade and destroy neighboring body tissue and spread to distant sites and cause death (for review, see Robins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-122). A tumor is said to have metastatized when it has spread from one organ or tissue to another.

A major problem in the chemotherapy of solid tumor cancers is delivery of therapeutic agents, such as drugs, in sufficient concentrations to eradicate tumor cells while at the same time minimizing damage to normal cells. Thus, studies in many laboratories are directed toward the design of biological delivery systems, such as antibodies, cytokines, and viruses for targeted delivery of drugs, pro-drug converting enzymes, and/or genes into tumor cells (see, *e.g.*, Crystal, R.G., 1995, Science 270:404-410).

2.1. CELLULAR IMMUNITY AND CYTOKINES

One strategy for the treatment of cancer involves enhancing or activating a cellular immune response. Successful induction of a cellular immune response directed toward autologous tumors offers several advantages over conventional chemotherapy: 1) immune recognition is highly specific, being directed exclusively toward tumors; 2) growth at metastatic sites can be suppressed through immune surveillance; 3) the diversity of immune response and recognition can compensate for different resistance mechanisms employed by tumor cells; 4) clonal expansion of cytotoxic T cells can occur more rapidly than the expanding tumor, resulting in antitumor mechanisms which ultimately overwhelm the tumor; and 5) a memory response can suppress disease recurrence in its earliest stages, prior to physical detection. Clinical studies of responding patients have borne out results from animal models demonstrating that successful immunotherapy involves the activation of CD8+ T cells (class I response), although evidence exists for participation of CD4+ T cells, macrophages, and NK cells. See, *e.g.*, Chapoval *et al.*, 1998, J. Immunol. 161:6977-6984; Gollub *et al.*, 1998, J. Clin. Invest. 102:561-575; Kikuchi et al., 1999, Int. J. Cancer 80:425-430; Pan et al., 1995, Int. J. Cancer 80:425-430; Saffran *et al.*, 1998, Cancer Gene Ther. 5:321-330; and Zimmermann *et al.*,1999, Eur. J. Immunol. 29:284-290.

2.2. TUMOR NECROSIS FACTOR (TNF) FAMILY OF CYTOKINES

The best characterized member of the TNF family is TNF-α. TNF-α is known to exert pleiotropic effects on the immune system. TNF-α is a cytokine which can exert potent cytotoxic effects directly on tumor cells. TNF-α is generally thought to exert its anti-tumor effects via other mechanisms such as stimulation of proliferation and differentiation, and prevention of apoptosis in monocytes (*see, e.g.*, Mangan *et al.*, 1991, J. Immunol. 146:1541-1546; and Ostensen *et al.*,1987, J. Immunol. 138:4185-4191), promotion of tissue factor-like procoagulant activity and suppression of endothelial cell surface anticoagulant activity, ultimately leading to clot formation within the tumor (reviewed in Beutler and Cerami, 1989, Ann. Rev. Immunol. 7:625-655; and Vassalli, P., 1992, Ann. Rev. Immunol.

10:411-452). However, as a result of these properties, systemic administration of TNF-α results in lethal consequences in the host due to disseminated intravascular coagulation.

Other cytokines have also been implicated in anti-tumor responses. IL-2 is a class I cytokine and is also thought to play a role in anti-tumor response. For example, spontaneously regressing melanomas have been associated with elevated intratumoral levels of TNF-α and IL-2. See, *e.g.*, Beutler and Cerami, 1989, Annu. Rev. Immunol. 7:625-655; Lowes *et al.*, 1997, J. Invest. Dermatol. 108:914-919; Mangan *et al.*, 1991, J. Immunol. 146:1541-1546; Scheruich *et al.*, 1987, J. Immunol. 138: 1786-1790.

Both TNF-α and IL-2 aid in lymphocyte homing, and IL-2 has been shown to induce tumor infiltration of natural killer (NK) cells, T-cells, and lymphokine activated killer (LAK) cells (see, *e.g.*, Etter *et al.*, 1998, Cytokine 10:395-403; Reinhardt *et al.*, 1997, Blood 89:3837-46; Chen *et al.*, 1997, J. Neuropathol. Exp. Neurol. 56:541-50; Vora *et al.*, 1996, Clin. Exp. Immunol. 105:155-62; Luscinskas *et al.*, 1996, J. Immunol. 157:326-35; Kjaergaard *et al.*, 1998, Scand. J. Immunol. 47, 532-540; Johansson *et al.*, 1996, Nat. Immun. 15:87-97; and Watanabe *et al.*, 1997, Am. J. Pathol. 150:1869-80). In the presence of both TNF-α and IL-2, the cytolytic activity of NK and LAK cells is increased, even when directed against TNF-insensitive cell lines (see, *e.g.* Ostensen *et al.*, 1987, J. Immunol. 138:4185-4191). However, therapeutic levels of IL-2 have also been shown to be toxic to the host.

Clearly, dose-limiting toxicity from systemic cytokine administration poses a significant barrier to realizing the potential of cytokines in cancer therapy. Moreover, systemic cytokine delivery can result in decreased homing of syngeneic T cells, thus opposing targeted immunotherapy, in addition to resulting in unwanted clinical side effects. See Addison *et al.*, 1998, Gene Ther. 5:1400-1409; Albertini *et al.*, 1997, Clin. Cancer Res. 3:1277-1288; Becker *et al.*, 1996, Proc. Natl. Acad. Sci. *USA* 93:7826-7831; Book *et al.*, 1998, J. Neuroimmunol. 92:50-59; Cao *et al.*, 1998, J. Cancer Res. Clin. Oncol. 124:88-92; D'Angelica *et al.*, 1999, Cancer Immunol. Immunother. 47:265-271; Deszo *et al.*, 1996, Clin. Cancer Res. 2:1543-1552; Kjaergaard *et al.*, 1998, Scand. J. Immunol. 47:532-540; Ostensen *et al.*, 1987, J. Immunol. 138:4185-4191; and Schirrmacher *et al.*, 1998, Clin. Cancer Res. 4:2635-2645.

2.3. DELIVERY OF CYTOKINES

Recent experimental animal and clinical studies have attempted to bypass systemic toxicity of cytokines and administer higher doses, through sub-systemic or alternative methods of delivery of cytokines. In murine models, sarcoma-180 tumors have been treated

with administration of a fusogenic liposome-encapsulated TNF-α gene, and systemic administration of polyethylene glycol-encapsulated TNF-α, which could localize to the tumor vasculature (see Tsutsumi *et al.*, 1996, Jpn. J. Cancer Res. 87:1078-1085). Sensitization of tumors to TNF-α by endothelial-monocyte-activating polypeptide II has also been reported (see, Marvin *et al.*, 1999, J. Surg. Res. 63:248-255; Wu *et al.*, 1996, Cancer Res.59:205-212).

In clinical studies, complete tumor eradication has been observed following high-dose TNF-α administration to patients via isolated limb perfusion, in combination with interferon-α or melphalan. However, this technique presents severe risks to the patient if the cytokines are not completely removed following treatment. Further, these treatments require limb isolation, which, in itself presents risks to the patient. See Eggermont *et al.*, 1997, Semin. Oncol. 24:547-555 Fraker et al., 1995, Cancer J. Sci. Am. 1:122-130; Lejeune *et al.*,1998, Curr. Opin. Immunol. 10:573-580; Marvin *et al.*, 1996, J. Surg. Res. 63:248-255; Mizuguchi *et al.*, 1998, Cancer Res. 58:5725-5730; Tsutsumi *et al.*, 1996, Jpn. J. Cancer Res. 87:1078-1085; and Wu *et al.*,1996, Cancer Res. 59, 205-212.

Carrier *et al*, 1992, J. Immunol. 148:1176-81 have reported the use of an attenuated *Salmonella* strain to deliver IL-1ß to mice. Saltzman has reported that the oral administration of an attenuated *Salmonella* strain containing interleukin-2 (IL-2) results in patterns of hepatic and splenic colonization of *Salmonella* and decreases MC-38 hepatic metastases (Saltzman *et al.*, 1997, Cancer Biother. Radiopharm. 12:37-45 and Saltzman *et al.*, 1997, J. Pediat. Surgery 32:301-306).

2.4. ANGIOGENESIS AND TUMORIGENESIS

Another strategy for the treatment of cancer involves the inhibition of angiogenesis. Angiogenesis is the process of growth of new capillaries from preexisting blood vessels. New capillaries are formed by a process in which the endothelial cells of the preexisting blood vessel, using proteolytic enzymes such as matrix metalloproteases, degrade the basement membranes in their vicinity, proliferate, migrate into surrounding stromal tissue and form microtubes . The process of angiogenesis is very tightly regulated by an interplay between negative and positive factors, and in adults is normally restricted to the female reproductive cycle and wound repair (Malonne *et al.*, 1999, Clin. Exp. Metastasis 17:1-14). Aberrant or abnormal regulation of angiogenesis has been implicated in many human disorders, including diabetic retinopathy, psoriasis, rheumatoid arthritis, cardiovascular disease, and tumorigenesis (Folkman, 1995, Nat. Med. 1:27-31).

Angiogenesis is a critical process for tumor growth and metastasis. Tumor formation is divided into two stages, the prevascular and vascular stages. Studies have shown that cells of prevascular tumors proliferate as rapidly as do cells from vascularized tumors. However, prevascular tumors rarely grow to more than 2-3 mm^3 because of the existence of an equilibrium between cell proliferation and cell death, the latter resulting from the hypoxic nature of the prevascular tumor (Folkman, 1995, Nat. Med. 1:27-31). The switch from the prevascular to vascular stage requires a shift in the balance of the regulatory factors of angiogenesis from a net balance favoring negative factors to one in which the positive factors, such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), predominate (Cao, 1998, Prog. Mol. Subcell. Biol. 20:161-176). The shift in balance between regulatory factors is a result of the up-regulation of the angiogenic factors and the simultaneous down-regulation of anti-angiogenic factors (Folkman, 1995, N. Eng. J. Med. 333:1757-1763).

2.5. ANTI-ANGIOGENIC FACTORS

Anti-angiogenic factors were postulated to exist on the basis of several related phenomena that led to the conclusion that primary tumors often inhibited the growth of their metastases (Cao, 1998, Prog. Mol. Subcell. Biol. 20:161-176). The first of these factors to be isolated was mouse angiostatin, a 38 kDa proteolytic fragment of plasminogen that is released into the circulation by primary Lewis lung carcinoma tumors and prevents the growth of secondary metastases (O'Reilly *et al.*, 1994, Cell 79:315-328). In humans, peptides of 40, 42 and 45 kDa produced by the limited proteolysis of plasminogen with metalloelastase have anti-angiogenic activity comparable to mouse angiostatin (O'Reilly *et al.*, 1994, Cell 79:315-328). Plasminogen itself has no such activity. It is also thought that tumor-associated macrophages are responsible for the production of angiostatin, since tumor cells themselves have no detectable angiostatin mRNA. Macrophage metalloelastase expression is induced by granulocyte colony stimulating factor (GM-CSF) secreted by the tumor cells (Dong *et al.*, 1997, Cell 88:801-810). In certain tumors, angiostatin production is catalyzed by serine proteases rather than metalloelastase, where serine proteases are produced directly by the tumor cells (Gately *et al.*, 1997, Cancer Res. 56:4887-4890). Administration of angiostatin at a concentration of 100mg/kg/day to experimental mice with primary tumors resulted in a strong inhibition of tumor growth without toxic side effects. The tumors regrew within 2 weeks of cessation of the angiostatin treatment, indicating that the tumors regress into a dormant state rather than completely die as a result of the treatment (O'Reilly *et al.*, 1996, Nat. Med. 2:689-692).

After the discovery of angiostatin, other angiogenesis inhibitors, including several angiogenesis-inhibiting peptides, were discovered and isolated. A more potent inhibitor of angiogenesis than angiostatin is kringle 5, a peptide comprising the fifth kringle domain of plasminogen (angiostatin comprises kringle domains 1-4). Kringle 5 can be produced by the proteolysis of plasminogen, and recombinant forms are also active (Cao *et al.*, 1997, J. Biol. Chem. 272:22924-22928).

Endostatin was isolated in a manner similar to the isolation of angiostatin (O'Reilly et al., 1997, Cell 88:1-20), the source being a murine hemangioendothelioma rather than a Lewis lung carcinoma. The peptide has an apparent molecular mass of 20 kDa whose sequence corresponds to the C-terminal of collagen XVIII (O'Reilly *et al.*, 1997, Cell 88:1-20), a region called NC1 that is divergent among various collagen molecules (Oh *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:4229-4233; and Rehn *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:4234-4238). In mice, the growth of Lewis lung carcinoma metastases is suppressed by the administration 0.3 mg/kg/day of recombinant endostatin, and the primary tumor regresses to a dormant state when the peptide is administered at 20 mg/kg/day. Functional recombinant endostatin can be produced from inclusion bodies, either *in vitro* by denaturation and refolding, or *in vivo* by the sustained release of subcutaneously administered endostatin inclusion body preparations (O'Reilly *et al.*, 1997, Cell 88:1-20). An alternative method of endostatin delivery consisting of intramuscular administration of an endostatin expression plasmid results in only the partial inhibition of tumor growth in a mouse model system (Blezinger *et al.*, 1999, Nat. Biotech. 17:343-348). Similarly, endostatin or angiotensin-encoding plasmids complexed to liposomes that were delivered intravenously resulted in a partial inhibition of tumor growth in a nude mouse model of breast cancer (Chen *et al.*, 1999, Cancer Res. 59:3308-3312).

Recently, a novel anti-angiogenic activity has been attributed to a C-terminal truncation peptide of the Serpin (Serine Protease Inhibitor) anti-thrombin (O'Reilly *et al.*, 1999, Science 285:1926-1928). Full length anti-thrombin has no inherent anti-angiogenic activity, but upon cleavage of the C-terminal reactive loop of the protein by thrombin, anti-thrombin acquires potent angiogenic activity. The proteolytic fragment is referred to hereinafter as anti-angiogenic anti-thrombin.

Other angiogenesis-inhibiting peptides known in the art include the 29 kDa N–terminal and a 40 kDa C-terminal proteolytic fragments of fibronectin (Homandberg *et al.*, 1985, J. Am. Pathol. 120:327-332); the 16 kDa proteolytic fragment of prolactin (Clapp *et al.*, 1993, Endocrinology 133:1292-1299); and the 7.8 kDa proteolytic fragment of platelet factor-4 (Gupta *et al.*, 1995, Proc. Natl. Acad. Sci. USA 92:7799-7803).

In addition to those naturally produced proteolytic fragments that have demonstrated anti-angiogenic effects, several synthetic peptides that correspond to regions of known extracellular matrix proteins have been assessed for activity in inhibiting angiogenesis. Synthetic peptides which have been demonstrated to be functional endothelial inhibitors, *i.e.* angiogenesis inhibitors, include a 13 amino acid peptide corresponding to a fragment of platelet factor-4 (Maione *et al.*, 1990, Cancer Res. 51:2077-2083); a 14 amino acid peptide corresponding to a fragment of collagen I (Tolma *et al.*, 1993, J. Cell Biol. 122:497-511); a 19 amino acid peptide corresponding to a fragment of Thrombospondin I (Tolsma *et al.*, 1993, J. Cell Biol. 122:497-511); and a 20 amino acid peptide corresponding to a fragment of SPARC (Sage *et al.*, 1995, J. Cell. Biochem. 57:1329-1334), a secreted cysteine-rich extracellular matrix glycoprotein whose expression in human melanoma cells leads to reduced cellular invasion *in vitro* and reduced tumorigenicity in an *in vivo* nude mouse model (Ledda et al., 1996, Nature Med. 3:171-176). Other peptides of less than 10 amino acids that inhibit angiogenesis and correspond to fragments of laminin, fibronectin, procollagen, and EGF have also been described (see the review by Cao, 1998, Prog. Mol. Subcell. Biol. 20:161-176).

The small fibronectin peptides that inhibit angiogenesis generally comprise the motif RGD. RGD is a peptide motif (amino acids Arg-Gly-Asp) used by proteins for recognition and binding to integrin molecules. The expression of integrin $\alpha_v\beta_3$ is associated with angiogenic blood vessels and inhibition of its activity by monoclonal antibodies blocks vascularization (Brooks *et al.*, 1994, Science 264:569-571). This has been confirmed by a study showing that the administration of cyclic pentapeptides containing the RGD motif inhibits the activity of vitronectin receptor-type integrins and block retinal neovascularization (Hammes *et al.*, 1996, Nature Medicine 2:529-533). The anti-angiogenic effect of integrin blockers such as cyclic pentapeptides and monoclonal antibodies has been shown to promote tumor regression by inducing the apoptosis of angiogenic blood vessels (Brooks *et al.*, 1994, Cell 79:1157-1164). Peptides comprising the RGD motif, and another integrin binding motif, NGR (amino acids Asn-Gln-Arg), showed markedly enhanced anti-tumor activity

The inhibition of the activity of another type of cell surface receptor, namely the urokinase plasminogen activator (uPA) receptor, also results in the inhibition of angiogenesis. The uPA receptor, upon ligand binding, initiates a proteolytic cascade that is necessary for the basement membrane invasion step of angiogenesis. Inhibition of the uPA receptor by receptor antagonists inhibits angiogenesis, tumor growth (Min *et al.*, 1996, Cancer Res. 56: 2428-2433) and metastasis (Crowley *et al.*, 1993, Proc. Natl. Acad. Sci.

USA 90:5021-5025). Such antagonists have been identified by bacteriophage peptide display of random peptides (Goodson *et al.*, Proc. Natl. Acad. Sci. USA 91:7129-7133). Dominant negative forms of the receptor's ligand, uPA, have also been identified (Min *et al.*, 1996, Cancer Res. 56: 2428-2433).

While the discovery of angiostatin, endostatin and other anti-angiogenic peptides provided an exciting new approach for cancer therapy, the reality of a course of treatment involving one or more of these peptides is the impracticality of the production of immense amounts of peptides (stemming from the cost and/or labor of having to produce, for an average person of 65 kg or 143 lbs, approximately 1.3 or 6.5 grams of protein per day, depending on the peptide) and the duration of the treatment (which has to be sustained if the tumor is to stay in regression). It is thought that the two main reasons that these peptides have to be administered in such large quantities are that, first, a majority are degraded in the blood stream and, second, of the molecules that do survive degradation only a very limited proportion make their way to the tumor. Thus, it would be a great advantage to the field of tumor therapy if anti-angiogenic proteins or peptides could be delivered more efficiently to the tumor and in a more cost-effective and patient-friendly manner.

2.6. BACTERIOCIN FAMILY

Colicin E3 (referred to hereinafter as ColE3) is a bacteriocin, *i.e.*, a bacterial proteinaceous toxin with selective activity, in that its host is immune to the toxin. Bacteriocins may be encoded by the host genome or by a plasmid, may have a broad or narrow range of hosts, and may have a simple structure comprising one or two subunits or may be a multi-subunit structure (Konisky, 1982, Ann. Rev. Microbiol. 36:125-144). In addition, a bacteriocin host has an immunity against the bacteriocin. The immunity is found in all cells of a given host population, even those that do not express the bacteriocin.

The cytotoxicity of ColE3 results from its inhibition of protein synthesis (Nomura, 1963, Cold Spring Harbor Symp. Quant. Biol. 28:315-324). The target of ColE3 activity is the 16S component of bacterial ribosomes, which is common to the 30S and 70S ribosomes (Bowman *et al.*, 1971, Proc. Natl. Acad. Sci. USA. 68:964-968), and the activity results in the degradation of the ribosome (Meyhack, 1970, Proc. Natl. Acad. Sci. USA). ColE3 activity is unique among RNAses, in that it does not cause the overall degradation of RNA, but cleaves mRNA molecules 49 nucleotides from the end, resulting in the separation of the rRNA from the mRNA and thereby inhibiting translation. The ribonuclease activity of ColE3 resides in the molecule itself, rather than being mediated by another protein

(Saunders, 1978, Nature 274:113-114). ColE3 is also able to penetrate the inner and outer membranes of the target cell.

In its naturally occurring form, ColE3 is a 60kDa protein complex consisting of a 50kDa and a 10kDa protein in a 1:1 ratio, the larger subunit having the nuclease activity and the smaller subunit having inhibitory function of the 50kDa subunit. Thus, the 50kDa protein acts as a cytotoxic protein (or toxin), and the 10kDa protein acts as an anti-toxin. The 50 kDa subunit comprises at least two functional domains, an N-terminal region required for translocation across target cell membranes, and a C-terminal region with catalytic (RNAse) activity. Within the host organism, the activity of the large subunit is inhibited by the small subunit. The subunits are thought to dissociate upon entry of the toxin into the target cell as a result of interaction with the target cell's outer membrane (reviewed by Konisky, 1982, Ann. Rev. Microbiol. 36:125-144).

The toxicity of the large subunit of ColE3 has been utilized to prevent the lateral spread of cloned genes among microorganisms. Diaz *et al.* (1994, Mol. Microbiol. 13:855-861) separated the two components of ColE3 such that the small (anti-toxic) subunit was expressed as a chromosomally integrated coding sequence and the large subunit was expressed from a plasmid. Bacteria with the chromosomally integrated small subunit are immune to plasmids that express the ColE3 large subunit, but if the plasmid were to be laterally transferred to another recipient that lacked the small subunit, that cell would be killed.

Colicin E3 (ColE3) has also been shown to have a profoundly cytotoxic effect on mammalian cells (see Smarda *et al.*, 1978, Folia Microbiol. 23:272-277), including a leukemia cell model system (see Fiska *et al.*, 1979, Experimentia 35:406-407). ColE3 activity targets the 40S subunit of the 80S mammalian ribosome (Turnowsky *et al.*, 1973, Biochem. Biophys. Res. Comm. 52:327-334).

2.7. BACTERIAL INFECTIONS AND CANCER

Early clinical observations reported cases in which certain cancers were reported to regress in patients with bacterial infections, See Nauts *et al.*, 1953, Acta Medica. Scandinavica 145:1-102, (Suppl. 276); and Shear, 1950, J.A.M.A. 142:383-390. Since these observations, Lee *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:1847-1851 (Lee *et al.*) and Jones *et al.*, 1992, Infect. Immun. 60:2475-2480 (Jones *et al.*) isolated mutants of *Salmonella typhimurium* that were able to invade HEp-2 (human epidermoid carcinoma) cells *in vitro* in significantly greater numbers than the wild-type strain. The "hyperinvasive" mutants were isolated under conditions of aerobic growth of the bacteria

that normally repress the ability of wild-type strains to invade HEp-2 animal cells. However, such hyperinvasive *Salmonella typhimurium* as described by Lee *et al.* and Jones *et al.* carry the risk of pan-invasive infection and could lead to wide-spread bacterial infection in the cancer patient.

Carswell *et al.*, 1975, Proc. Natl. Acad. Sci. USA 72:3666-3669, demonstrated that mice injected with bacillus Calmette-Guerin (BCG) have increased serum levels of TNF and that TNF-positive serum caused necrosis of the sarcoma Meth A and other transplanted tumors in mice. As a result of such observations, immunization of cancer patients with BCG injections is currently utilized in some cancer therapy protocols. See Sosnowski, 1994, Compr. Ther. 20:695-701; Barth and Morton, 1995, Cancer 75 (Suppl. 2):726-734; Friberg, 1993, Med. Oncol. Tumor. Pharmacother. 10:31-36 for reviews of BCG therapy.

However, TNF-α-mediated septic shock is among the primary concerns associated with bacteria, and can have toxic or lethal consequences for the host (Bone, 1992, JAM 268:3452-3455; Dinarello *et al.*, 1993, JAMA 269:1829-1835). Further, dose-limiting, systemic toxicity of TNF-α has been the major barrier to effective clinical use. Modifications which reduce this form of an immune response would be useful because TNF-α levels would not be toxic.

2.8. TUMOR-TARGETED BACTERIA

Genetically engineered *Salmonella* have been demonstrated to be capable of tumor targeting, possess anti-tumor activity and are useful in delivering effector genes such as the herpes simplex thymidine kinase (HSV TK) to solid tumors (Pawelek *et al.*, WO 96/40238).

2.9. DECREASED INDUCTION OF TNF-α BY MODIFIED BACTERIAL LIPID A

Modifications to the lipid composition of tumor-targeted bacteria which alter the immune response as a result of decreased induction of TNFα production were suggested by Pawelek *et al.* (Pawelek *et al.*, WO 96/40238). Pawelek *et al.* provided methods for isolation of genes from *Rhodobacter* responsible for monophosphoryl lipid A (MLA) production. MLA acts as an antagonist to septic shock. Pawelek *et al.* also suggested the use of genetic modifications in the lipid A biosynthetic pathway, including the mutation *firA*, which codes for the third enzyme UDP-3-O (R-30 hydroxylmyristoyl)-glucosamine –acyltransferase in lipid A biosynthesis (Kelley *et al.*, 1993, J. Biol. Chem. 268:19866-19874). Pawelek *et al.* showed that mutations in the *firA* gene induce lower levels of TNFα.

In *Escherichia coli*, the gene *msbB* (*mlt*) which is responsible for the terminal myristalization of lipid A has been identified (Engel, *et al.*, 1992, J. Bacteriol. 174:6394-6403; Karow and Georgopoulos 1992, J. Bacteriol. 174:702-710; Somerville *et al.*, 1996, J. Clin. Invest. 97:359-365). Genetic disruption of this gene results in a stable non-conditional mutation which lowers TNFα induction (Somerville *et al.*, 1996, J. Clin. Invest. 97:359-365; Somerville, WO 97/25061). These references, however, do not suggest that disruption of the *msbB* gene in tumor-targeted *Salmonella* would result in bacteria which are less virulent and more sensitive to chelating agents.

The problems associated with the use of bacteria as gene delivery vectors center on the general ability of bacteria to directly kill normal mammalian cells as well as their ability to overstimulate the immune system via TNFα which can have toxic consequences for the host (Bone, 1992, JAMA 268:3452-3455; and Dinarello *et al.*, 1993, JAMA 269:1829-1835). In addition to these factors, resistance to antibiotics can severely complicate coping with the presence of bacteria within the human body (Tschape, 1996, D T W Dtsch Tierarztl Wochenschr 1996 103:273-7; Ramos *et al.*, 1996, Enferm Infec. Microbiol. Clin. 14: 345-51).

Hone and Powell, WO97/18837 ("Hone and Powell"), disclose methods to produce gram-negative bacteria having non-pyrogenic Lipid A or LPS. Although Hone and Powell broadly asserts that conditional mutations in a large number of genes including *msbB*, *kdsA*, *kdsB*, *kdtA*, and *htrB*, etc. can be introduced into a broad variety of gram-negative bacteria including *E. coli*, *Shigella* sp., *Salmonella* sp., etc., the only mutation exemplified is an *htrB* mutation introduced into *E. coli*. Further, although Hone and Powell propose the therapeutic use of non-pyrogenic *Salmonella* with a mutation in the *msbB* gene, there is no enabling description of how to accomplish such use. Moreover, Hone and Powell propose using non-pyrogenic bacteria only for vaccine purposes.

The objective of a vaccine vector is significantly different from the tumor-targeted bacteria utilized in the compositions and methods of the invention described herein. Thus, vaccine vectors have requirements quite different from tumor-targeted bacteria. Vaccine vectors are intended to elicit an immune response. A preferred live bacterial vaccine must be immunogenic so that it elicits protective immunity; however, the vaccine must not be capable of excessive growth *in vivo* which might result in adverse reactions. According to the teachings of Hone and Powell, a suitable bacterial vaccine vector is temperature sensitive having minimal replicative ability at normal physiological ranges of body temperature.

Maskell, WO98/33923, describes a mutant strain of *Salmonella* having a mutation in the *msbB* gene which induces TNFα at a lower level as compared to a wild type strain.

Bermudes *et al.*, WO 99/13053, teach compositions and methods for the genetic disruption of the *msbB* gene in *Salmonella*, which results in *Salmonella* possessing a lesser ability to elicit TNFα and reduced virulence compared to the wild type. In certain embodiments, some such mutant *Salmonella* have increased sensitivity to chelating agents as compared to wild type *Salmonella*. See also, Low *et al.*, 1999, Nature Biotech. 17:37-47.

Citation or identification of any reference in Section 2, or any section of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention encompasses treatment protocols that provide a better therapeutic effect than currently existing clinical therapies for solid tumor cancers. In particular, the present invention provides combination therapies for the treatment of a solid tumor cancer in a subject comprising administering to said subject one or more immunomodulatory agents and one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria. The present invention also provides combination therapies for the treatment of a solid tumor cancer in a subject comprising administering to said subject one or more immunomodulatory agents and one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, wherein said tumor-targeted bacteria comprise one or more nucleic acid molecules encoding one or more therapeutic molecules.

The combination of one or more immunomodulatory agents and one or more tumor-targeted bacteria produces a better therapeutic effect in a subject with a solid tumor cancer than that of either treatment alone. In certain embodiments, the combination of one or more immunomodulatory agents and one or more tumor-targeted bacteria achieves a 20%, preferably a 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% better therapeutic effect in a subject with a solid tumor cancer than either treatment alone. In particular embodiments, the combination of one or more immunomodulatory agents and one or more tumor-targeted bacteria achieves a 20%, preferably a 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% greater reduction in the growth of a solid tumor cancer or the volume of a solid tumor cancer in a subject than either treatment alone. In other embodiments, the combination of one or more immunomodulatory agents and one or more tumor-targeted

bacteria has a more than additive or synergistic therapeutic effect in a subject with a solid tumor cancer. The combination therapies of the invention enable lower dosages and/or less frequent of dosing of tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, to be administered to a subject with a solid tumor cancer to achieve a therapeutic effect. The combination therapies of the invention improve the efficacy of tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, in the treatment of solid tumor cancers, while avoiding or limiting the adverse or unwanted side effects associated with the administration of bacteria to subjects whose immune system has been altered or compromised in some aspect.

The present invention provides methods for the enhanced delivery of one or more therapeutic molecules to a solid tumor cancer in a subject comprising administering to said subject one or more immunomodulatory agents and one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, comprising nucleic acid molecules encoding one or more therapeutic molecules. The methods of the present invention permit lower dosages and/or less frequent dosing of tumor-targeted bacteria to be administered to a subject with a solid tumor cancer to achieve a therapeutically effective amount of one or more therapeutic molecules at the site of the solid tumor cancer.

In one embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of one or more attenuated tumor-targeted bacteria, wherein the attenuated tumor-targeted bacteria is a facultative anaerobe or facultative aerobe. In accordance with this embodiment, the attenuated tumor-targeted bacteria may comprise or be engineered to comprise one or more nucleic acid molecules encoding one or more therapeutic molecules. Examples of facultative anaerobes and facultative aerobes which may be used in accordance with the methods of the invention include, but are not limited to, *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Yersinia enterocohtica*, *Listeria monocytogenies*, *Mycoplasma hominis*, and *Streptococcus* sp. Preferably, the gram-negative tumor-targeted bacteria are attenuated by introducing one or more mutations in one or more genes in the lipopolysaccharide (LPS) biosynthetic pathway, and optionally one or more mutations to auxotrophy for one or more nutrients or metabolites. The attenuated tumor-targeted bacteria induce lower levels of tumor necrosis factor-α (TNF-α) than their wild-type counterpart, and thus, avoid or reduce the risk of inducing septic shock in a subject with a solid tumor cancer when administered to said subject in accordance with the methods of the invention.

In another embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of one or more attenuated or non-pathogenic tumor-targeted bacteria, wherein the tumor-targeted bacteria is an obligate anaerobe. In accordance with this embodiment, the tumor-targeted bacteria may comprise or be engineered to comprise one or more nucleic acid molecules encoding one or more therapeutic molecules. Examples of obligate anaerobes which may be used in accordance with the methods of the invention include, but are not limited to, *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp., *Peptostreptococcus* sp. and any spores thereof.

In one embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of one or more attenuated tumor-targeted bacteria, wherein the attenuated tumor-targeted bacteria is *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Yersinia enterocohtica*, *Listeria monocytogenies*, *Mycoplasma hominis*, or *Streptococcus* sp. In another embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of one or more attenuated tumor-targeted bacteria, wherein the attenuated tumor-targeted bacteria is *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Yersinia enterocohtica*, *Listeria monocytogenies*, *Mycoplasma hominis*, or *Streptococcus* sp. and the tumor-targeted bacteria comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a specific embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of one or more attenuated or non-pathogenic tumor-targeted bacteria, wherein the tumor-targeted bacteria is *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp., or *Peptostreptococcus* sp, or any spores thereof. In another embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of one or more attenuated or non-pathogenic tumor-targeted bacteria, wherein the tumor-targeted bacteria is *Clostridium* sp., *Actinomyces* sp.,

Bifidobacterium sp., *Propionibacterium* sp., or *Peptostreptococcus* sp., or any spores thereof and the tumor-targeted bacteria comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a preferred embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of one or more attenuated tumor-targeted *Salmonella*. In another preferred embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of one or more attenuated tumor-targeted *Salmonella*, wherein the attenuated tumor-targeted *Salmonella* comprises one or more nucleic acid molecules encoding one or more therapeutic molecules. Examples of *Salmonella* sp. which may be used in accordance with the invention include, but are not limited to, *Salmonella typhi*, *Salmonella choleraesuis*, or *Salmonella enteritidis*. Preferably, the tumor-targeted *Salmonella* are attenuated by introducing one or more mutations in one or more genes in the lipopolysaccharide (LPS) biosynthetic pathway, and optionally one or more mutations to auxotrophy for one or more nutrients or metabolites. In a preferred embodiment, the attenuated tumor-targeted *Salmonella* comprises a genetically modified *msbB* gene, expresses an altered lipid A molecule compared to wild-type *Salmonella* sp., and induces TNF-α expression at a level less than that induced by a wild-type *Salmonella* sp. The growth of an attenuated tumor-targeted *Salmonella* used in accordance with the invention may be sensitive to a chelating agent such as, *e.g.*, Ethylenediaminetetraacetic Acid (EDTA), Ethylene Glycol-bis(β-aminoethyl Ether) N, N, N', N'-Tetraacetic Acid (EGTA), or sodium citrate. For example, a chelating agent may inhibit the growth of an attenuated tumor-targeted *Salmonella* by about 90%, 95%, 99%, or 99.5% compared to the growth of a wild-type *Salmonella* sp. Preferably, the attenuated tumor-targeted *Salmonella* used in accordance with the invention survive in macrophages at about 50% to about 30%, about 30% to about 10%, or about 10% to about 1% of the level of survival of a wild-type *Salmonella* sp.

In a preferred embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of VNP20009 (a.k.a., YS1646 or 41.2.9; deposited with the American Type Culture Collection (ATCC) and assigned

Accession No. 202165) or YS1456 (a.k.a., 8.7; deposited with the ATCC and assigned Accession No. 202164). In another preferred embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of VNP20009 or YS1456, wherein VNP20009 or YS1456 are engineered to express one or more nucleic acid molecules encoding one or more therapeutic molecules.

The present invention provides methods of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said methods comprising administering to said subject an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity and an effective amount of one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria. The present invention also provides methods of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said methods comprising administering to said subject an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity and an effective amount of one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, wherein the tumor-targeted bacteria comprise one or more therapeutic molecules.

In one embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity and an effective amount of one or more attenuated tumor-targeted bacteria, wherein the attenuated tumor-targeted bacteria is *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Yersinia enterocohtica*, *Listeria monocytogenies*, *Mycoplasma hominis*, or *Streptococcus* sp.. In another embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity and an effective amount of one or more attenuated tumor-targeted bacteria, wherein the attenuated tumor-targeted bacteria is *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Yersinia enterocohtica*, *Listeria monocytogenies*, *Mycoplasma hominis*, or *Streptococcus* sp. and the tumor-targeted bacteria comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In another embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity and an effective amount of one or more attenuated or non-pathogenic tumor-targeted bacteria, wherein the tumor-targeted bacteria is *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp., *Peptostreptococcus* sp., or any spores thereof. In another embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity and an effective amount of one or more attenuated or non-pathogenic tumor-targeted bacteria, wherein the tumor-targeted bacteria is *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp., *Peptostreptococcus* sp., or any spores thereof and the tumor-targeted bacteria comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a preferred embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity and an effective amount of one or more attenuated tumor-targeted *Salmonella*. In another preferred embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity and an effective amount of one or more attenuated tumor-targeted *Salmonella*, wherein the attenuated tumor-targeted *Salmonella* comprises one or more nucleic acid molecules encoding one or more therapeutic molecules. Preferably, the tumor-targeted *Salmonella* are attenuated by introducing one or more mutations in one or more genes in the lipopolysaccharide (LPS) biosynthetic pathway, and optionally one or more mutations to auxotrophy for one or more nutrients or metabolites. In a preferred embodiment, the attenuated tumor-targeted *Salmonella* comprises a genetically modified *msbB* gene, expresses an altered lipid A molecule compared to wild-type *Salmonella* sp., and induces TNF-α expression at a level less than that induced by a wild-type *Salmonella* sp.

In another preferred embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said

method comprising administering to said subject an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity and an effective amount of VNP20009 (a.k.a., YS1646 or 41.2.9; deposited with the American Type Culture Collection (ATCC) and assigned Accession No. 202165) or YS1456 (a.k.a., 8.7; deposited with the ATCC and assigned Accession No. 202164). In yet another preferred embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity and an effective amount of VNP20009 or YS1456, wherein VNP20009 or YS1456 comprise one or more nucleic acid molecules encoding one or more therapeutic molecules.

Suitable dosage ranges for the tumor-targeted bacteria are generally from about 1.0 c.f.u./kg to about 1×10^{10} c.f.u./kg; optionally from about 1.0 c.f.u./kg to about 1×10^{8} c.f.u./kg; optionally from about 1×10^{2} c.f.u./kg to about 1×10^{8} c.f.u./kg; optionally from about 1×10^{4} c.f.u./kg to about 1×10^{8} c.f.u./kg; and optionally from about 1×10^{4} c.f.u./kg to about 1×10^{10} c.f.u./kg. Suitable dosages for the tumor-targeted bacteria also include ranges from about 1×10^{6} c.f.u./m^2 to about 5×10^{9} c.f.u./m^2. In a specific embodiment, a suitable dosage for the tumor-targeted bacteria is 3×10^{8} c.f.u./m^2.

In accordance with the invention, one or more immunomodulatory agents are administered to a subject with a solid tumor cancer to reduce or inhibit one or more aspects of the immune response. Any technique well-known to one skilled in the art can be used to measure one or more aspects of the immune response in a particular subject, and thereby determine when it is appropriate to administer an immunomodulatory agent and a tumor-targeted bacteria to said subject in accordance with the methods of the invention. In a preferred embodiment, a mean absolute lymphocyte count of approximately 500 cells/mm^3, preferably 600 cells/mm^3, 650 cells/mm^3, 700 cells/mm^3, 750 cells/mm^3, 800 cells/mm^3, 900 cells/mm^3, 1000 cells/mm^3, 1100 cells/mm^3, or 1200 cells/mm^3 is maintained in a subject. In another preferred embodiment, a subject with a solid tumor cancer is not administered an immunomodulatory agent if his/her mean absolute lymphocyte count is 500 cells/mm^3 or less, 550 cells/mm^3 or less, 600 cells/mm^3 or less, 650 cells/mm^3 or less, 700 cells/mm^3 or less, 750 cells/mm^3 or less, 800 cells/mm^3 or less, or 900 cells/mm^3 or less, 1000 cells/mm^3 or less.

In accordance with the methods of the invention, one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, are administered to a subject with a

solid tumor cancer prior to (*e.g*, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 45 minutes, 60 minutes, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 12 hours, 14 hours, 16 hours, 18 hours, 20 hours, 22 hours, 24 hours, 2 days, 4 days, 5 days, 7 days or 2 weeks before), concomitantly with, subsequent to (*e.g.*, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 45 minutes, 60 minutes, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 12 hours, 14 hours, 16 hours, 18 hours, 20 hours, 22 hours, 24 hours, 2 days, 4 days, 5 days, 7 days or 2 weeks after) the administration of one or more immunomodulatory agents. Preferably, the administration of the immunomodulatory agents in combination with the administration of the tumor-targeted bacteria increases the efficacy of the tumor-targeted bacteria, while limiting the adverse or unwanted side effects associated with the administration of bacteria to a subject whose immune system is altered or compromised in one or more aspects. Examples of immunomodulatory agents include, but are not limited to, methotrexate, leflunomide, cyclosporin A, methylprednisolone, corticosteroids, mycophenolate mofetil, rapamycin, mizoribine, deoxyspergualin, macrolide antibiotics, cytokines (*e.g.*, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-15, IFN alpha, IFN beta and IFN gamma), anti-T cell receptor antibodies (*e.g.*, anti-CD3 antibodies and anti-CD8 antibodies), a soluble T cell receptor, B cell receptor antibodies, anti-cytokine antibodies (*e.g.*, anti-IL2 antibodies, anti-IL-4 antibodies, anti-IL-10 antibodies, anti-IL-12 antibodies, and anti-IFN alpha antibodies, anti-IFN beta antibodies and anti-IFN gamma antibodies), a soluble cytokine receptor, and anti-cytokine receptor antibodies (*e.g.*, anti-IL-2R antibodies).

In certain embodiments, the immunomodulatory agent used in accordance with the methods of the invention is a chemotherapeutic agent that is administered to a subject at a dosage less than those used in standard chemotherapeutic therapies for the treatment of cancer. Suitable dosage ranges for the immunomodulatory agents are generally from about 1×10^{-4} to about $1 \times 10^{+3}$ mpk (a.k.a., mg per kg of a subject's body weight). In particular embodiments, the immunomodulatory agent used in accordance with the methods of the invention is a chemotherapeutic agent that is administered to a subject at dosages analogous to dosages used to treat autoimmune disorders or organ transplant rejection. In other embodiments, the immunomodulatory agent used in accordance with the methods of the invention is not a chemotherapeutic agent, but rather an agent other than a chemotherapeutic agent such as *e.g.*, a cytokine, an anti-cytokine antibody, an anti-cytokine receptor antibody, a T cell receptor antibody, a soluble T cell receptor, a B cell receptor antibody, or a soluble cytokine receptor.

In a preferred embodiment, the immunomodulatory agent used in accordance with the methods of the invention is an anti-CD8 antibody, methotrexate, or cyclosporin A. In

another preferred embodiment, the immunomodulatory agents used in accordance with the methods of the invention comprise a combination of methotrexate and cyclosporin A.

In a preferred embodiment, the tumor-targeted bacteria (preferably, attenuated tumor-targeted bacteria) used in accordance with the invention comprise one or more nucleic acid molecules encoding one or more therapeutic molecules. The tumor-targeted bacteria facilitate the localized delivery of therapeutic molecules which may cause adverse side effects when administered systemically to a subject with a solid tumor cancer. The therapeutic molecules may have therapeutic effects themselves or improve the therapeutic effects of the tumor-targeted bacteria. Examples of therapeutic molecules include, but are not limited to, anti-angiogenic factors, cytostatic factors and cytotoxic factors. In another embodiment, the tumor-targeted bacteria (preferably, attenuated tumor-targeted bacteria) used in accordance with the invention comprise one or more nucleic acid molecules encoding one or more fusion proteins, wherein the fusion proteins comprise one or more therapeutic molecules fused to one or more heterologous peptides, polypeptides, or proteins.

The present invention provides methods for treating a solid tumor cancer in a subject, said methods comprising administering to a subject in need thereof, one or more tumor-targeted bacteria, one or more immunomodulatory agents, and at least one other known cancer therapy, wherein the tumor-targeted bacteria optionally comprise nucleotide sequences encoding one or more therapeutic molecules. In a specific embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering an effective amount of one or more tumor-targeted bacteria, preferably attenuated-tumor-targeted bacteria, an effective amount of one or more immunomodulatory agents, and an effective amount of at least one other anti-cancer agent such as a chemotherapeutic agent, wherein the tumor-targeted bacteria is a facultative aerobe or facultative anaerobe. In another specific embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering an effective amount of one or more tumor-targeted bacteria, preferably attenuated-tumor-targeted bacteria, an effective amount of one or more immunomodulatory agents, and an effective amount of at least one other anti-cancer agent such as a chemotherapeutic agent, wherein the tumor-targeted bacteria is a facultative aerobe or facultative anaerobe and comprises one or more nucleic acid molecules encoding one or more therapeutic agents. Examples of facultative aerobes and facultative aerobes include, but are not limited to,

Escherichia coli, *Salmonella* sp., *Shigella* sp., *Yersinia enterocohtica*, *Listeria monocytogenies*, *Mycoplasma hominis*, or *Streptococcus* sp.

In another embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering an effective amount of one or more tumor-targeted bacteria, preferably attenuated or non-pathogenic tumor-targeted bacteria, an effective amount of one or more immunomodulatory agents, and an effective amount of at least one other anti-cancer agent such as a chemotherapeutic agent, wherein the tumor-targeted bacteria is an obligate anaerobe. In another specific embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering an effective amount of one or more tumor-targeted bacteria, preferably attenuated or non-pathogenic tumor-targeted bacteria, an effective amount of one or more immunomodulatory agents, and an effective amount of at least one other anti-cancer agent such as a chemotherapeutic agent, wherein the tumor-targeted bacteria is an obligate anaerobe and comprises one or more nucleic acid molecules encoding one or more therapeutic agents. Examples of obligate anaerobes include, but are not limited to, *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp., *Peptostreptococcus* sp. and any spores thereof.

In a specific embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering an effective amount of one or more tumor-targeted bacteria, preferably attenuated-tumor-targeted bacteria, an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, peptides with immunomodulatory activity, and an effective amount of at least one other anti-cancer agent such as a chemotherapeutic agent, wherein the tumor-targeted bacteria is a facultative aerobe or facultative anaerobe. In another specific embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering an effective amount of one or more tumor-targeted bacteria, preferably attenuated-tumor-targeted bacteria, an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, peptides with immunomodulatory activity, and an effective amount of at least one other anti-cancer agent such as a chemotherapeutic agent, wherein the tumor-targeted bacteria is a facultative aerobe or facultative anaerobe and comprises one or more nucleic acid molecules encoding one or more therapeutic agents.

In another embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering an effective amount of one or more tumor-targeted bacteria, preferably attenuated-tumor-targeted bacteria, an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, peptides with immunomodulatory activity, and an effective amount of at least one other anti-cancer agent such as a chemotherapeutic agent, wherein the tumor-targeted bacteria is an obligate anaerobe. In another specific embodiment, the present invention provides a method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering an effective amount of one or more tumor-targeted bacteria, preferably attenuated-tumor-targeted bacteria, an effective amount of one or more nucleic acid molecules encoding proteins, polypeptides, peptides with immunomodulatory activity, and an effective amount of at least one other anti-cancer agent such as a chemotherapeutic agent, wherein the tumor-targeted bacteria is an obligate anaerobe and comprises one or more nucleic acid molecules encoding one or more therapeutic agents.

The invention provides pharmaceutical compositions comprising pharmaceutically acceptable carriers, one or more immunomodulatory agents, and one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria. The invention also provides pharmaceutical compositions comprising pharmaceutically acceptable carriers, one or more immunomodulatory agents, and one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, comprising nucleotide sequences encoding one or more therapeutic molecules. The pharmaceutical compositions of the invention may be used in accordance with the methods of the invention for the treatment of a solid tumor cancer in a subject. Preferably, the tumor-targeted bacteria are attenuated by introducing one or more mutations in one or more genes in the lipopolysaccharide (LPS) biosynthetic pathway, and optionally one or more mutations to auxotrophy for one or more nutrients or metabolites.

In one embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated tumor-targeted bacteria, wherein said attenuated tumor-targeted bacteria is a facultative anaerobe or facultative aerobe. In another embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated tumor-targeted bacteria, wherein said attenuated tumor-targeted bacteria is a facultative anaerobe or facultative aerobe and comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a specific embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated tumor-targeted bacteria, wherein the attenuated tumor-targeted bacteria is *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Yersinia enterocohtica*, *Listeria monocytogenies*, *Mycoplasma hominis*, or *Streptococcus* sp.. In another specific embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated tumor-targeted bacteria, wherein the attenuated tumor-targeted bacteria is *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Yersinia enterocohtica*, *Listeria monocytogenies*, *Mycoplasma hominis*, or *Streptococcus* sp. and the tumor-targeted bacteria comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In another embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated tumor-targeted bacteria, wherein said attenuated tumor-targeted bacteria is an obligate anaerobe. In another embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated tumor-targeted bacteria, wherein said attenuated tumor-targeted bacteria is an obligate anaerobe and comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a specific embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated or non-pathogenic tumor-targeted bacteria, wherein the tumor-targeted bacteria is *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp., *Peptostreptococcus* sp., or any spores thereof. In another specific embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated or non-pathogenic tumor-targeted bacteria, wherein the tumor-targeted bacteria is *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp., *Peptostreptococcus* sp., or any spores thereof and the tumor-targeted bacteria comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a preferred embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated tumor-targeted *Salmonella*. In another preferred embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated tumor-targeted *Salmonella*, wherein

said attenuated tumor-targeted *Salmonella* comprise one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a preferred embodiment, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated tumor-targeted *Salmonella*, wherein the attenuation of the tumor-targeted *Salmonella* is due, at least in part, to one or more mutations in the *msbB* gene. In another preferred embodiment, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more attenuated tumor-targeted *Salmonella*, wherein the attenuated tumor-targeted *Salmonella* comprise one or more nucleic acid molecules encoding one or more therapeutic molecules and the attenuation of the tumor-targeted *Salmonella* is due, at least in part, to one or more mutations in the *msbB* gene.

In another preferred embodiment, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and VNP20009 (a.k.a., YS1646 or 41.2.9; deposited with the American Type Culture Collection (ATCC) and assigned Accession No. 202165) or YS1456 (a.k.a., 8.7; deposited with the ATCC and assigned Accession No. 202164). In yet another preferred embodiment, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and VNP20009 or YS1456, wherein VNP20009 or YS1456 are engineered to express one or more nucleic acid molecules encoding one or more therapeutic molecules.

The present invention provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria. The invention also provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more tumor-targeted bacteria, preferably tumor-targeted bacteria, wherein the tumor-targeted bacteria comprise one or more nucleic acid molecules encoding one or more therapeutic molecules.

In one embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated

tumor-targeted bacteria, wherein said attenuated tumor-targeted bacteria is a facultative anaerobe or facultative aerobe. In another embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated tumor-targeted bacteria, wherein said attenuated tumor-targeted bacteria is a facultative anaerobe or facultative aerobe and comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In another embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated tumor-targeted bacteria, wherein said attenuated tumor-targeted bacteria is an obligate anaerobe. In another embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated tumor-targeted bacteria, wherein said attenuated tumor-targeted bacteria is an obligate anaerobe and comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a specific embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated tumor-targeted bacteria, wherein the attenuated tumor-targeted bacteria is *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Yersinia enterocohtica*, *Listeria monocytogenies*, *Mycoplasma hominis*, or *Streptococcus* sp.. In another specific embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated tumor-targeted bacteria, wherein the attenuated tumor-targeted bacteria is *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Yersinia enterocohtica*, *Listeria monocytogenies*, *Mycoplasma hominis*, or *Streptococcus* sp. and the tumor-targeted bacteria comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a specific embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated or non-pathogenic tumor-targeted bacteria, wherein the tumor-targeted bacteria is *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp.,

Peptostreptococcus sp., or any spores thereof. In another specific embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated or non-pathogenic tumor-targeted bacteria, wherein the tumor-targeted bacteria is *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp., *Peptostreptococcus* sp., or any spores thereof and the tumor-targeted bacteria comprises one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a preferred embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated tumor-targeted *Salmonella*. In another preferred embodiment, a pharmaceutical composition comprises a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated tumor-targeted *Salmonella*, wherein said attenuated tumor-targeted *Salmonella* comprise one or more nucleic acid molecules encoding one or more therapeutic molecules.

In a preferred embodiment, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated tumor-targeted *Salmonella*, wherein the attenuation of the tumor-targeted *Salmonella* is due, at least in part, to one or more mutations in the *msbB* gene. In another preferred embodiment, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and one or more attenuated tumor-targeted *Salmonella*, wherein the attenuated tumor-targeted *Salmonella* comprise one or more nucleic acid molecules encoding one or more therapeutic molecules and the attenuation of the tumor-targeted *Salmonella* is due, at least in part, to one or more mutations in the *msbB* gene.

In another preferred embodiment, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and VNP20009 (a.k.a., YS1646 or 41.2.9; deposited with the American Type Culture Collection (ATCC) and assigned Accession No. 202165) or

YS1456 (a.k.a., 8.7; deposited with the ATCC and assigned Accession No. 202164). In yet another preferred embodiment, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier, one or more nucleic acid molecules encoding one or more proteins, polypeptides, or peptides with immunomodulatory activity, and VNP20009 or YS1456, wherein VNP20009 or YS1456 are engineered to express one or more nucleic acid molecules encoding one or more therapeutic molecules.

The invention also provides a pharmaceutical pack or kit comprising one or more containers with one or more of the components of the pharmaceutical compositions of the invention. The kit further comprises instructions for use of the composition. In certain embodiments of the invention, the kit comprises a document providing instructions for the use of the composition of the invention in, *e.g.*, written and/or electronic form. Said instructions provide information relating to, *e.g.*, dosage, methods of administration, and duration of treatment. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

In one embodiment, a kit of the invention comprises an immunomodulatory agent contained in a first vial, an attenuated tumor-targeted bacteria contained in a second vial, and instructions for administering the immunomodulatory agent and attenuated tumor-targeted bacteria to a subject with a solid tumor cancer, wherein the attenuated tumor-targeted bacteria is a facultative anaerobe or facultative aerobe. In another embodiment, a kit of the invention comprises an immunomodulatory agent contained in a first vial, an attenuated tumor-targeted bacteria contained in a second vial, and instructions for administering the immunomodulatory agent and attenuated tumor-targeted bacteria to a subject with a solid tumor cancer, wherein the attenuated tumor-targeted bacteria is an anaerobe. In accordance with these embodiments, the attenuated tumor-targeted bacteria included in the kit may comprise one or more nucleic acid molecules encoding one or more therapeutic molecules. In a preferred embodiment, a kit of the invention comprises an immunomodulatory agent contained in a first vial, an attenuated tumor-targeted *Salmonella* contained in a second vial, and instructions for administering the immunomodulatory agent and attenuated tumor-targeted *Salmonella* to a subject with a solid tumor cancer. In accordance with this embodiment, the attenuated tumor-targeted *Salmonella* included in the kit may comprise one or more nucleic acid molecules encoding one or more therapeutic molecules.

3.1. DEFINITIONS

As used herein, *Salmonella* encompasses all *Salmonella* species, including: *Salmonella typhi*, *Salmonella choleraesuis*, and *Salmonella enteritidis*. Serotypes of *Salmonella* are also encompassed herein, for example, *typhimurium*, a subgroup of *Salmonella enteritidis*, commonly referred to as *Salmonella typhimurium*.

Analog: As used herein, the term "analog" in the context of "an analog of a therapeutic molecule" or "an analog of an immunomodulatory agent which is a proteinaceous agent (*e.g.*, an immunomodulatory polypeptide) " refers to a polypeptide that possesses a similar or identical function as a therapeutic molecule or an immunomodulatory polypeptide but does not necessarily comprise a similar or identical amino acid sequence of a therapeutic molecule or an immunomodulatory polypeptide, or possesses a similar or identical structure of a therapeutic molecule or an immunomodulatory polypeptide. A polypeptide that has a similar amino acid sequence refers to a polypeptide that satisfies at least one of the following: (a) a polypeptide having an amino acid sequence that is at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% identical to the amino acid sequence of a therapeutic molecule or an immunomodulatory polypeptide; (b) a polypeptide encoded by a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence encoding a therapeutic molecule or an immunomodulatory polypeptide described herein of at least 5 contiguous amino acid residues, at least 10 contiguous amino acid residues, at least 15 contiguous amino acid residues, at least 20 contiguous amino acid residues, at least 25 contiguous amino acid residues, at least 40 contiguous amino acid residues, at least 50 contiguous amino acid residues, at least 60 contiguous amino residues, at least 70 contiguous amino acid residues, at least 80 contiguous amino acid residues, at least 90 contiguous amino acid residues, at least 100 contiguous amino acid residues, at least 125 contiguous amino acid residues, or at least 150 contiguous amino acid residues; and (c) a polypeptide encoded by a nucleotide sequence that is at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% identical to the nucleotide sequence encoding a therapeutic molecule or an immunomodulatory polypeptide. A polypeptide with a similar structure to a therapeutic molecule or an immunomodulatory polypeptide refers to a polypeptide that has a similar secondary, tertiary or quaternary structure to the therapeutic molecule or the immunomodulatory polypeptide. The structure of a polypeptide can be determined by methods known to those skilled in the art, including but not limited to,

peptide sequencing, X-ray crystallography, nuclear magnetic resonance, circular dichroism, and crystallographic electron microscopy.

As used herein, the term "analog" in the context of "an immunomodulatory agent" which is a non-proteinaceious agent refers to an organic or inorganic compound that possesses a similar or identical function to an immunomodulatory agent and that is structurally similar to an immunomodulatory agent.

Antibody: As used herein, the terms "antibody" and "antibodies" refer to monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies, camelised antibodies, chimeric antibodies, single-chain Fvs (scFv), single chain antibodies, Fab fragments, F(ab') fragments, disulfide-linked Fvs (sdFv), and anti-idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site. Immunoglobulin molecules can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass.

Anti-neoplastic: As used herein, the term "anti-neoplastic activity" refers to an agent including, but not limited to, a protein, polypeptide, peptide, nucleic acid, small molecule, and organic or inorganic compound, that has cytostatic or cytotoxic activity. Preferably, a therapeutic molecule with anti-neoplastic activity inhibits or reduces the growth of a tumor or tumor cells, reduces the volume of a tumor, kills tumor cells, or inhibits or reduces the spread of tumor cells (metastasis).

Attenuation: Attenuation is a modification so that tumor-targeted bacteria are less pathogenic. The end result of attenuation is that the risk of toxicity as well as other side-effects is decreased, when the tumor-targeted bacteria are administered to an animal.

Derivative: As used herein, the term "derivative" in the context of a "derivative of a therapeutic molecule" or "derivative of an immunomodulatory agent" which is a proteinaceious agent (*e.g.*, an immunomodulatory polypeptide)" refers to a polypeptide that comprises an amino acid sequence of a therapeutic molecule or an immunomodulatory polypeptide, which has been altered by the introduction of amino acid residue substitutions, deletions or additions, or by the covalent attachment of any type of molecule to the polypeptide. For example, but not by way of limitation, a therapeutic molecule or an immunomodulatory polypeptide may be modified, *e.g.*, by proteolytic cleavage, linkage to a cellular ligand or other protein, etc. A derivative of a therapeutic molecule or an immunomodulatory polypeptide may be modified by chemical modifications using

techniques known to those of skill in the art (*e.g.*, by acylation, phosphorylation, carboxylation, glycosylation, selenium modification and sulfation). Further, a derivative of a therapeutic molecule or an immunomodulatory polypeptide may contain one or more non-classical amino acids. A polypeptide derivative possesses a similar or identical function as a therapeutic molecule or an immunomodulatory polypeptide.

As used herein, the term "derivative" in the context of a "derivative of an immunomodulatory" which is a non-proteinaceious agent refers to an organic or inorganic compound that is formed based upon the structure of an immunomodulatory agent. A derivative of an immunomodulatory agent includes, but is not limited to, an immunomodulatory agent that is modified, *e.g.*, by the addition or deletion of hydroxyl or carboxyl groups. A derivative of an immunomodulatory agent also includes an immunomodulatory agent that is esterified, alkylated, and/or phosphorylated. A derivative of an immunomodulatory agent possesses a similar or identical function as the immunomodulatory agent from which it was derived.

Fragment: As used herein, the term "fragment" refers to a peptide or polypeptide comprising an amino acid sequence of at least 2 contiguous amino acid residues, at least 5 contiguous amino acid residues, at least 10 contiguous amino acid residues, at least 15 contiguous amino acid residues, at least 20 contiguous amino acid residues, at least 25 contiguous amino acid residues, at least 40 contiguous amino acid residues, at least 50 contiguous amino acid residues, at least 60 contiguous amino residues, at least 70 contiguous amino acid residues, at least contiguous 80 amino acid residues, at least contiguous 90 amino acid residues, at least contiguous 100 amino acid residues, at least contiguous 125 amino acid residues, at least 150 contiguous amino acid residues, at least contiguous 175 amino acid residues, at least 200 contiguous amino acid residues, or at least contiguous 250 amino acid residues of the amino acid sequence of a therapeutic molecule or an immunomodulatory agent. In a specific embodiment, a fragment of a therapeutic molecule retains at least one function of the therapeutic molecule. In a preferred embodiment, a fragment of a therapeutic molecule retains the anti-neoplastic and/or anti-angiogenic activity of the therapeutic molecule. In another specific embodiment, a fragment of an immunomodulatory agent retains at least one function of the immunomodulatory agent.

Functional fragment: As used herein, the term "functional fragment" refers to a fragment of a therapeutic molecule or an immunomodulatory agent that retains at least one function of said therapeutic molecule or immunomodulatory agent, respectively.

Fusion protein: As used herein, the term "fusion protein" refers to a polypeptide that comprises an amino acid sequence of a first protein, or functional fragment, analog or derivative thereof, and an amino acid sequence of a heterologous protein (*i.e.*, a second protein, or functional fragment, analog or derivative thereof different than the first protein, or functional fragment, analog or derivative thereof). In one embodiment, a fusion protein comprises a therapeutic molecule fused to a heterologous peptide, polypeptide, or protein. In accordance with this embodiment, the heterologous peptide, polypeptide or protein may or may not be a second, different therapeutic molecule. In a specific embodiment, a fusion protein comprises a polypeptide, peptide or protein with anti-neoplastic and/or anti-angiogenic activity and a heterologous protein (*e.g.* a different second therapeutic molecule or a release factor). In accordance with this embodiment, a polypeptide, protein or peptide with anti-neoplastic and/or anti-angiogenic activity can be any therapeutic molecule described herein, or known to one of skill in the art.

Gene products: As used herein, the term "gene products" refers to RNA molecules (*e.g.*, mRNA), proteins, polypeptides and peptides.

Immunomodulatory agent: As used herein, the term "immunomodulatory agent" and variations thereof, including but not limited to immunomodulant or immunomodulatory drug, refer to an agent that modulates a subject's immune system. In particular, an immunomodulatory agent is an agent that alters the ability of a subject's immune system to respond to one or more foreign antigens. In a specific embodiment, an immunomodulatory agent is an agent that shifts one aspect of a subject's immune response. In a preferred embodiment of the invention, an immunomodulatory agent is an agent that inhibits or reduces a subject's immune system (*i.e.*, an immunosuppressant agent). Preferably, an immunomodulatory agent that inhibits or reduces a subject's immune system inhibits or reduces the ability of a subject's immune system to respond to one or more foreign antigens. In certain embodiments, immunomodulatory agents do not include chemotherapeutic agents such as methotrexate, cyclosporin A, leflunomide, cisplatin, ifosfamide, paclitaxol, taxanes, topoisomerase I inhibitors (*e.g.*, CPT-11, topotecan, 9-AC, and GG-211), gemcitabine, vinorelbine, oxaliplatin, 5-fluorouracil (5-FU), leucovorin, vinorelbine, temodal, taxol, cytochalasin B, gramicidin D, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, melphalan, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin homologs, and cytoxan.

Immunospecifically binds to an antigen: As used herein, the term "immunospecifically binds to an antigen" and analogous terms refer to peptides, polypeptides, proteins, fusion proteins and antibodies or fragments thereof that specifically bind to an antigen or a fragment and do not specifically bind to other antigens. A peptide, polypeptide, protein, or antibody that immunospecifically binds to an antigen may bind to other peptides, polypeptides, or proteins with lower affinity as determined by, *e.g.*, immunoassays, BIAcore, or other assays known in the art. Antibodies or fragments that immunospecifically bind to an antigen may cross-reactive with related antigens. Preferably, antibodies or fragments that immunospecifically bind to an antigen do not cross-react with other antigens.

Isolated: As used herein, an "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. Preferably, an "isolated" nucleic acid molecule is free of sequences (preferably protein encoding sequences) which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. In certain embodiments, the term "isolated"as used herein when referring to a nucleic acid molecule does not include an isolated chromosome.

An "isolated" polypeptide is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of heterologous protein (also referred to herein as a "contaminating protein"). When the protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, 10%, or 5% of the volume of the protein preparation. When the protein is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, *i.e.*, it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. Accordingly such preparations of the protein have

less than about 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or compounds other than the polypeptide of interest.

Nucleic Acids: As used herein, the terms "nucleic acids", "nucleic acid molecules" and "nucleotide sequences" include DNA molecules (*e.g.*, cDNA or genomic DNA), RNA molecules (*e.g.*, mRNA), combinations of DNA and RNA molecules or hybrid DNA/RNA molecules, and analogs of DNA or RNA molecules. Such analogs can be generated using, for example, nucleotide analogs, which include, but are not limited to, inosine or tritylated bases. Such analogs can also comprise DNA or RNA molecules comprising modified backbones that lend beneficial attributes to the molecules such as, for example, nuclease resistance or an increased ability to cross cellular membranes. The nucleic acids or nucleotide sequences can be single-stranded, double-stranded, may contain both single-stranded and double-stranded portions, and may contain triple-stranded portions, but preferably is double-stranded DNA. In one embodiment, the nucleotide sequences comprise a contiguous open reading frame encoding a therapeutic molecule or fragment thereof, *e.g.*, a cDNA molecule. In another embodiment, the nucleotide sequences comprise a contiguous open reading frame encoding an immunomodulatory agent or fragment thereof, *e.g.*, a cDNA molecule.

Side effects: As used herein, the term "side effects" encompasses unwanted and adverse effects of a therapeutic molecule. Adverse effects are always unwanted, but unwanted effects are not necessarily adverse. An adverse effect from a therapeutic molecule might be harmful or uncomfortable or risky. Examples of adverse side effects include, but are not limited to, fever, nausea, vomiting, the chills, myelosuppression, alopecia, body weight loss, and septic shock.

Subject: As used herein, the term "subject" refers to an animal including, but not limited to, a mammal (*e.g.*, a farm animal such as a cow, a pig, and a horse, a domestic animal such as a cat and a dog, and a human), and a bird (*e.g.*, a chicken). In a specific embodiment, the term "subject" refers to a mammal. In a preferred embodiment, the term "subject" refers to a human. In certain embodiments, a subject is an animal with a solid tumor cancer which is refractory to radiation or chemotherapy.

Tumor-targeted: As used herein, the term "tumor-targeted" in the context of facultative anaerobes or facultative aerobes that are tumor-targeted bacteria refers to the ability to preferentially localize to a cancerous target cell or tissue relative to a non-cancerous counterpart cell or tissue and/or preferentially replicate in the target cell cancer or tumor environment. Thus, a tumor-targeted bacteria such as *Salmonella* preferentially attaches to, infects and/or remains viable and replicates in the cancerous

target cell or the tumor environment. In certain embodiments of the invention, a tumor-targeted bacteria preferentially replicates in a cancerous target cell or tumor environment relative to a non-cancerous counterpart cell or tissue. In certain embodiments, a tumor-targeted bacteria undergoes at least 2 rounds, at least 4 rounds, at least 5 rounds, at least 8 rounds, at least 10 rounds, at least 15 rounds, at least 20 rounds, or more rounds of replication at physiological temperatures in a cancerous target cell or tumor environment. As used herein, the term "tumor-targeted" in the context of obligate anaerobes that are tumor-targeted bacteria refers to the ability to preferentially localize to a hypoxic area associated with a tumor or tumor environment and/or preferentially replicate in the hypoxic tumor cell environment.

Therapeutic molecule: As used herein, the terms "therapeutic molecule", "therapeutic agent" and variations thereof refer to a molecule that inhibits or reduces the growth of a tumor or tumor cells, reduces the volume of a tumor, kills tumor cells, inhibits or reduces the spread of tumor cells (metastasis), or ameliorates one or more symptoms associated with a cancer. A therapeutic molecule may be proteinaceous, a nucleic acid molecule, a small organic molecule, or a product of an enzymatic reaction. In a specific embodiment, a therapeutic molecule is a fusion protein. In certain embodiments, a therapeutic molecule is an immunomodulatory agent. In other embodiments, a therapeutic molecule is not an immunomodulatory agent. Preferably, a therapeutic molecule utilized in accordance with the invention is useful for the treatment of sarcomas, lymphomas that clinicians would characterize as solid tumors, carcinomas, or other solid tumor cancers.

Therapeutically effective amount: As used herein, terms "therapeutically effective amount" and "an effective amount" refer to the amount of an agent sufficient to result in the desired therapeutic effect. Examples of suitable dosages of effective amounts of agents are given *infra* in Section 5.5. With regard to therapeutic molecules, the term "therapeutically effective amount" refers to the amount of one or more therapeutic molecules sufficient to inhibit or reduce the growth of a tumor or tumor cells, reduce the volume of a tumor, kill tumor cells, inhibit or reduce the spread of tumor cells (metastasis), ameliorate one or more symptoms associated with a cancer, or to increase the therapeutic effect of one or more tumor-targeted bacteria in a subject with a solid tumor cancer. With regard to tumor-targeted bacteria, the term "therapeutically effective amount" refers to the amount of one or more tumor-targeted bacteria sufficient to inhibit or reduce the growth of a tumor or tumor cells, reduce the volume of a tumor, kill tumor cells, inhibit or reduce the spread of tumor cells (metastasis), ameliorate one or more symptoms associated with a cancer, or deliver an effective amount of one or more therapeutic molecules to the site of a solid tumor cancer in

a subject. With regard to immunomodulatory agents, the term "therapeutically effective amount" refers to the amount of one or more immunomodulatory agents sufficient to increase the therapeutic effect of one or more tumor-targeted bacteria in a subject with a solid tumor cancer, or to enhance the localization and/or replication of the tumor-targeted bacteria in the subject in a cancerous cell or tumor environment.

Treatment of a solid tumor: As used herein, the terms "treatment of a solid tumor", "treatment of a solid tumor cancer", "treat a solid tumor", "treating a solid tumor cancer", and "treat a solid tumor cancer" encompass inhibiting or reducing the growth of a tumor or tumor cells, reducing the volume of a tumor, killing tumor cells, inhibiting or reducing the spread of tumor cells (metastasis), or ameliorating one or more symptoms associated with a solid tumor cancer.

Virulence: Virulence is a relative term describing the general ability to cause disease, including the ability to kill normal cells or the ability to elicit septic shock which is a state of internal organ failure due to a complex cytokine cascade, initiated by TNFα.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 demonstrates the ability of the VNP20009 *Salmonella* to target B16-F10 melanoma tumors in the presence of the immunomodulatory agent methotrexate.

FIG. 2 demonstrates the ability of the immunomodulatory agent methotrexate to augment the ability of the VNP20009 *Salmonella* to target Cloudman melanoma tumors in DBA/2 mice.

FIG. 3 demonstrates the ability of the VNP20009 *Salmonella* strain to target B16-F10 melanoma tumors in mice in the presence of immunomodulatory antibodies specific to CD4 and CD8a.

FIG. 4 demonstrates the ability of an immunomodulatory agent such as methotrexate to increase the therapeutic effect of the VNP20009 *Salmonella* strain on B16-F10 melanoma.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides improved methods for treating a solid tumor cancer in a subject comprising administering to a subject with a solid tumor cancer one or more immunomodulatory agents and tumor-targeted bacteria. The present invention provides methods for treating a solid tumor cancer in a subject comprising administering to said subject one or more immunomodulatory agents prior to, concomitantly with, or subsequent to the administration of tumor-targeted bacteria. The present invention provides methods

for treating a solid tumor cancer in a subject comprising administering to said subject one or more doses of a therapeutically effective amount of one or more tumor-targeted bacteria, and one or more doses of a therapeutically effective amount of one or more immunomodulatory agents. In accordance with the methods of the invention, a subject with a solid tumor cancer may be administered repeated doses of tumor-targeted bacteria and/or immunomodulatory agents as part of a therapeutic protocol to treat cancer. The tumor-targeted bacteria and/or immunomodulatory agents can be administered locally and/or systemically. In specific embodiments of the invention, the immunomodulatory agents and the tumor-targeted bacteria can be administered separately or as an admixture. In a preferred embodiment, the tumor-targeted bacteria used in accordance with the invention are attenuated.

The methods of the invention improve the efficacy of tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, in the treatment of solid tumor cancers, while avoiding or limiting the adverse or unwanted side effects associated with the administration of bacteria to subjects whose immune system has been altered or compromised in some aspect by an immunomodulatory agent. The methods of the present invention enable lower dosages and/or less frequent dosing of tumor-targeted bacteria to be administered to a subject with a solid tumor cancer to achieve a therapeutic effect. In particular, the administration of an immunomodulatory agent in combination with said tumor-targeted bacteria reduces the dosage of tumor-targeted bacteria necessary to inhibit or reduce the growth of a tumor or tumor cells, reduce the volume of a tumor, kill tumor cells, inhibit or reduce the spread of tumor cells (metastasis), or ameliorate one or more symptoms associated with a cancer. Without intending to be limited as to the mechanism, the administration of one or more immunomodulatory agents in combination with the administration of tumor-targeted bacteria to a subject with a solid tumor cancer may suppress the immune response of said subject, enabling a lower dosage of the tumor-targeted bacteria to be administered to the subject to achieve a concentration of tumor-targeted bacteria in said subject which is therapeutically effective.

In a specific embodiment of the invention, the tumor-targeted bacteria used in accordance with the methods of the invention are themselves effective therapeutics against neoplastic growths. In another embodiment of the invention, the therapeutic effect derived from administering tumor-targeted bacteria in combination with one or more immunomodulatory agents to a subject with a solid tumor cancer results from the delivery of one or more therapeutic molecules to a tumor site by the tumor-targeted bacteria. In yet another embodiment, the therapeutic effect derived from administering tumor-targeted

bacteria in combination with one or more immunomodulatory agents to a subject with a solid tumor cancer results from the bacteria themselves and the delivery of one or more therapeutic molecules to the tumor site by the bacteria.

In certain embodiments, the present invention provides methods and compositions for the enhanced delivery of one or more therapeutic molecules to a solid tumor cancer. In particular, the present invention provides methods for the enhanced delivery of one or more therapeutic molecules to a solid tumor cancer in a subject comprising administering to said subject one or more immunomodulatory agents and tumor-targeted bacteria (*e.g.*, *Salmonella*) expressing one or more therapeutic molecules. Preferably, attenuated tumor-targeted bacteria are utilized to deliver one or more therapeutic molecules locally to a tumor site. The localized delivery of therapeutic molecules reduces the potential side effects associated with systemic delivery of certain therapeutic molecules (*e.g.*, septic shock caused by TNF-α). The present invention provides improved methods for the efficient local delivery of high levels of one or more therapeutic molecules, which may be toxic or induce unwanted or adverse side effects when delivered systemically to a subject.

In certain embodiments, the invention provides methods for the enhanced delivery of one or more therapeutic molecules comprising administering one or more immunomodulatory agents and tumor-targeted bacteria (*e.g.*, *Salmonella*), preferably attenuated tumor-targeted bacteria, as a vector for the delivery of one or more therapeutic molecules to an appropriate site of action, *e.g.*, the site of a solid tumor. In a specific embodiment, the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, used in the compositions and methods of the invention are facultative aerobes or facultative anaerobes. In another embodiment, the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, used in the compositions and methods of the invention are obligate anaerobes. In preferred embodiments, the tumor-targeted bacteria encode or may be engineered to encode one or more therapeutic molecules. In a preferred embodiment, the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, used in accordance with the methods of the invention are *Salmonella* mutants. In a preferred embodiment, one or more doses of immunomodulatory agents are administered to a subject with a solid tumor cancer who has received or is receiving tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, as needed to facilitate the therapeutic effect of the tumor-targeted bacteria or the therapeutic molecules expressed by the tumor-targeted bacteria.

The invention also provides methods for the delivery of at least two different therapeutic molecules to a tumor environment in a subject, said methods comprising

administering to said subject one or more immunomodulatory agents and one or more tumor-targeted bacteria expressing at least two different therapeutic. The different therapeutic molecules can be produced by the same strain or different strains of tumor-targeted bacteria. Different strains of tumor-targeted bacteria, preferably different strains of attenuated tumor-targeted bacteria, can be administered to a subject with a solid tumor cancer concomitantly with each other or subsequent to each other. One or more doses of one or more immunomodulatory agents can be administered to a subject with a solid tumor cancer prior to, concomitantly with, subsequent to the administration of each tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria.

The present invention also provides methods for local delivery of one or more fusion proteins by tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, to the site of a solid tumor(s). In particular, the present invention provides methods for delivering one or more fusion proteins to a subject with a solid tumor cancer comprising administering to said subject one or more immunomodulatory agents (preferably, non-fusion proteins) and tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, engineered to express one or more fusion proteins. In one embodiment, tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, are engineered to express a fusion protein comprising a signal peptide and a therapeutic molecule. In another embodiment, tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, are engineered to express a fusion protein comprising a signal peptide, a proteolytic cleavage site, and a therapeutic molecule. In another embodiment, tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, are engineered to express a fusion protein comprising a ferry peptide and a therapeutic molecule. In another embodiment, tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, are engineered to express a fusion protein comprising a signal peptide, a ferry peptide and a therapeutic molecule. In yet another embodiment, tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, are engineered to express a fusion protein comprising a signal peptide, a proteolytic cleavage site, a ferry peptide and a therapeutic molecule. In accordance with the invention, tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, engineered to express one or more fusion proteins and one or more non-fusion-protein therapeutic molecules can be administered in combination with one or more immunomodulatory agents.

The present invention provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more tumor-targeted bacteria. In a specific embodiment of the invention, the pharmaceutical composition comprises tumor-targeted bacteria that themselves have a

therapeutic effect in the treatment of a solid tumor cancer. The present invention also provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier, one or more immunomodulatory agents, and one or more tumor-targeted bacteria comprising one or more nucleic acid molecules encoding one or more therapeutic molecules. Further, the present invention provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier, one or more immunomodulatory agents and one or more tumor-targeted bacteria engineered to contain one or more nucleic acid molecules encoding one or more fusion proteins and/or one or more non-fusion-protein therapeutic molecules. Preferably, the tumor-targeted bacteria included in the pharmaceutical compositions of the invention are attenuated.

The compositions of the invention are useful in methods of the treatment of solid tumor cancers in a subject. In particular, the compositions of the invention are useful for the treatment of solid tumors, including, but not limited to, sarcomas, carcinomas, lymphomas that clinicians would characterize as solid tumors, and other solid tumor cancers, including but not limited to, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, central nervous system cancer, germ cell line cancer, melanoma, renal cancer, bladder cancer, and mesothelioma. In a specific embodiment of the invention, the subject that the compositions of the invention are administered to is an animal with a solid tumor cancer. In a preferred embodiment, the subject that the compositions of the invention are administered to is a mammal (*e.g.*, a dog, a cat, a horse, a cow, a monkey, or a pig) with a solid tumor cancer. In a more preferred embodiment, the subject that the compositions of the invention are administered to is a human being with a solid tumor cancer.

The invention also provides a pharmaceutical pack or kit comprising one or more containers with one or more of the components of the pharmaceutical compositions of the invention. The kit further comprises instructions for use of the composition. In certain embodiments of the invention, the kit comprises a document providing instructions for the use of the composition of the invention in, *e.g.*, written and/or electronic form. Said instructions provide information relating to, *e.g.*, dosage, methods of administration, and duration of treatment. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

In one embodiment, a kit of the invention comprises an immunomodulatory agent contained in a first vial, tumor-targeted bacteria contained in a second vial, and instructions for administering the immunomodulatory agent and the tumor-targeted bacteria to a subject with a solid tumor cancer, wherein the attenuated tumor-targeted bacteria is a facultative anaerobe or facultative aerobe. In another embodiment, a kit of the invention comprises an immunomodulatory agent contained in a first vial, tumor-targeted bacteria contained in a second vial, and instructions for administering the immunomodulatory agent and the tumor-targeted bacteria to a subject with a solid tumor cancer, wherein the attenuated tumor-targeted bacteria is an obligate anaerobe. In accordance with these embodiments, the tumor-targeted bacteria included in the kit may comprise one or more nucleic acid molecules encoding one or more therapeutic molecules. Preferably, the tumor-targeted bacteria included in the kit is attenuated. In a preferred embodiment, a kit of the invention comprises an immunomodulatory agent contained in a first vial, tumor-targeted *Salmonella* contained in a second vial, and instructions for administering the immunomodulatory agent and the tumor-targeted *Salmonella* to a subject with a solid tumor cancer. In accordance with this embodiment, the tumor-targeted *Salmonella* included in the kit may comprise one or more nucleic acid molecules encoding one or more therapeutic molecules. Preferably, the tumor-targeted *Salmonella* included in the kit is attenuated.

The kits of the invention may also comprise a means of testing the effectiveness of the pharmaceuticals of the invention. Said means of testing the effectiveness of the pharmaceuticals of the invention include, but are not limited to, tumorigenic cell lines, means of conducting a biopsy procedure, means for administering tumorigenic cells to an animal model, molecular markers (*e.g.*, antibodies and probes for *in situ* hybridization) for testing the expression of therapeutic molecules, molecular markers (*e.g.*, antibodies and probes for *in situ* hybridization) for testing the activity state of the immune system *etc.* Optionally, associated with such a kit can be a description of how to conduct said tests.

For reasons of clarity, the detailed description is divided into the following subsections: Immunomodulatory Agents; Tumor-Targeted Bacteria; Therapeutic Molecules; Expression Vehicles; Methods and Compositions for Delivery; Methods of Determining the Therapeutic Utility; and Kits.

5.1. IMMUNOMODULATORY AGENTS

Any immunomodulatory agent well-known to one of skill in the art may be used in the methods and compositions of the invention. Immunomodulatory agents can affect one or more or all aspects of the immune response in a subject. Aspects of the immune

response include, but are not limited to, the inflammatory response, the complement cascade, leukocyte and lymphocyte differentiation, proliferation, and/or effector function, monocyte and/or basophil counts, and the cellular communication among cells of the immune system. In certain embodiments of the invention, an immunomodulatory agent modulates one aspect of the immune response. In other embodiments, an immunomodulatory agent modulates more than one aspect of the immune response. In a preferred embodiment of the invention, the administration of an immunomodulatory agent to a subject inhibits or reduces one or more aspects of the subject's immune response capabilities. In a specific embodiment of the invention, the immunomodulatory agent inhibits or suppresses the immune response in a subject.

Immunomodulatory agents include, but are not limited to, agents capable of inhibiting: (i) the formation of neutralizing antibodies directed against the tumor-targeted bacteria by activated B cells; (ii) the elimination of the tumor-targeted bacteria by cytolytic T lymphocytes (CTLs); (iii) the activation and/or replication of T cells, including T helper cells; and (iv) the innate immune response. An immunomodulatory agent may be selected to interfere with the interactions between the T helper subsets (TH1 or TH2) and B cells to inhibit neutralizing antibody formation. An immunomodulatory agent may be selected to inhibit the interaction between TH1 cells and CTLs to reduce the occurrence of CTL-mediated elimination of the tumor-targeted bacteria. An immunomodulatory agent may be selected to alter (*e.g.*, inhibit or suppress) the proliferation, differentiation, or function of the $CD4^+$ and/or $CD8^+$ T cells. For example, antibodies specific for T cells can be used as immunomodulatory agents to deplete, or alter the proliferation, differentiation, or function of $CD4^+$ and/or $CD8^+$ T cells.

Examples of immunomodulatory agents include, but are not limited to, proteinaneous agents such as cytokines, peptide mimetics, and antibodies (*e.g.*, human, humanized, chimeric, monoclonal, polyclonal, Fvs, ScFvs, Fab or F(ab)2 fragments or epitope binding fragments), small molecules, organic compounds, and inorganic compounds. In particular, immunomodulatory agents include, but are not limited to, methothrexate, leflunomide, cyclophosphamide, cyclosporin A, and macrolide antibiotics (*e.g.*, FK506 (tacrolimus)), methylprednisolone (MP), corticosteroids, mycophenolate mofetil, rapamycin (sirolimus), mizoribine, deoxyspergualin, brequinar, malononitriloamindes (*e.g.*, leflunamide), anti-T cell receptor antibodies (*e.g.*, anti-CD4 antibodies (*e.g.*, cM-T412 (Boeringer), IDEC-CE9.1® (IDEC and SKB), mAB 4162W94, Orthoclone and OKTcdr4a (Janssen-Cilag)), anti-CD3 antibodies (*e.g.*, Nuvion (Product Design Labs), OKT3 (Johnson & Johnson), or Rituxan (IDEC)), anti-CD5 antibodies (*e.g.*,

an anti-CD5 ricin-linked immunoconjugate), anti-CD7 antibodies (*e.g.*, CHH-380 (Novartis)), anti-CD8 antibodies (Pharmingen), anti-CD40 ligand monoclonal antibodies (*e.g.*, IDEC-131 (IDEC)), anti-CD52 antibodies (*e.g.*, CAMPATH 1H (Ilex)), anti-CD2 antibodies, anti-CD11a antibodies (*e.g.*, Xanelim (Genentech)), and anti-B7 antibodies (*e.g.*, IDEC-114) (IDEC))), CTLA4-immunoglobulin LFA3TIP (Biogen), cytokines (*e.g.*, interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, TNF-α, TNF-β, interferon (IFN)-α, IFN-β, IFN-γ, and GM-CSF), anti-cytokine receptor antibodies (*e.g.*, anti-IFN receptor antibodies, anti-IL-2 receptor antibodies (*e.g.*, Zenapax (Protein Design Labs)), anti-IL-4 receptor antibodies, anti-IL-6 receptor antibodies, anti-IL-10 receptor antibodies, and anti-IL-12 receptor antibodies), and anti-cytokine antibodies (*e.g.*, anti-IFN antibodies, anti-TNF-α antibodies, anti-IL-1β antibodies, anti-IL-6 antibodies, anti-IL-8 antibodies (*e.g.*, ABX-IL-8 (Abgenix)), anti-IL-9 antibodies, and anti-IL-12 antibodies).

In a preferred embodiment, proteins, polypeptides or peptides (including antibodies) that are utilized as immunomodulatory agents are derived from the same species as the recipient of the proteins, polypeptides or peptides so as to reduce the likelihood of an immune response to those proteins, polypeptides or peptides. In another preferred embodiment, when the subject is a human, the proteins, polypeptides, or peptides that are utilized as immunomodulatory agents are human or humanized.

In accordance with the invention, one or more immunomodulatory agents are administered to a subject with a solid tumor cancer to reduce or inhibit one or more aspects of the immune response. Any technique well-known to one skilled in the art can be used to measure one or more aspects of the immune response in a particular subject, and thereby determine when it is appropriate to administer an immunomodulatory agent in combination with a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria) to said subject in accordance with the invention. In a preferred embodiment, a mean absolute lymphocyte count of approximately 500 cells/mm^3, preferably 600 cells/mm^3, 650 cells/mm^3, 700 cells/mm^3, 750 cells/mm^3, 800 cells/mm^3, 900 cells/mm^3, 1000 cells/mm^3, 1100 cells/mm^3, or 1200 cells/mm^3 is maintained in a subject. In another preferred embodiment, a subject with a solid tumor cancer is not administered an immunomodulatory agent if his/her mean absolute lymphocyte count is 500 cells/mm^3 or less, 550 cells/mm^3 or less, 600 cells/mm^3 or less, 650 cells/mm^3 or less, 700 cells/mm^3 or less, 750 cells/mm^3 or less, 800 cells/mm^3 or less, 900 cells/mm^3 or less or 1000 cells/mm^3 or less.

In accordance with the invention, one or more immunomodulatory agents are administered to a subject with a solid tumor cancer prior to, subsequent to, or

response include, but are not limited to, the inflammatory response, the complement cascade, leukocyte and lymphocyte differentiation, proliferation, and/or effector function, monocyte and/or basophil counts, and the cellular communication among cells of the immune system. In certain embodiments of the invention, an immunomodulatory agent modulates one aspect of the immune response. In other embodiments, an immunomodulatory agent modulates more than one aspect of the immune response. In a preferred embodiment of the invention, the administration of an immunomodulatory agent to a subject inhibits or reduces one or more aspects of the subject's immune response capabilities. In a specific embodiment of the invention, the immunomodulatory agent inhibits or suppresses the immune response in a subject.

Immunomodulatory agents include, but are not limited to, agents capable of inhibiting: (i) the formation of neutralizing antibodies directed against the tumor-targeted bacteria by activated B cells; (ii) the elimination of the tumor-targeted bacteria by cytolytic T lymphocytes (CTLs); (iii) the activation and/or replication of T cells, including T helper cells; and (iv) the innate immune response. An immunomodulatory agent may be selected to interfere with the interactions between the T helper subsets (TH1 or TH2) and B cells to inhibit neutralizing antibody formation. An immunomodulatory agent may be selected to inhibit the interaction between TH1 cells and CTLs to reduce the occurrence of CTL-mediated elimination of the tumor-targeted bacteria. An immunomodulatory agent may be selected to alter (*e.g.*, inhibit or suppress) the proliferation, differentiation, or function of the $CD4^+$ and/or $CD8^+$ T cells. For example, antibodies specific for T cells can be used as immunomodulatory agents to deplete, or alter the proliferation, differentiation, or function of $CD4^+$ and/or $CD8^+$ T cells.

Examples of immunomodulatory agents include, but are not limited to, proteinaneous agents such as cytokines, peptide mimetics, and antibodies (*e.g.*, human, humanized, chimeric, monoclonal, polyclonal, Fvs, ScFvs, Fab or F(ab)2 fragments or epitope binding fragments), small molecules, organic compounds, and inorganic compounds. In particular, immunomodulatory agents include, but are not limited to, methothrexate, leflunomide, cyclophosphamide, cyclosporin A, and macrolide antibiotics (*e.g.*, FK506 (tacrolimus)), methylprednisolone (MP), corticosteroids, mycophenolate mofetil, rapamycin (sirolimus), mizoribine, deoxyspergualin, brequinar, malononitriloamindes (*e.g.*, leflunamide), anti-T cell receptor antibodies (*e.g.*, anti-CD4 antibodies (*e.g.*, cM-T412 (Boeringer), IDEC-CE9.1® (IDEC and SKB), mAB 4162W94, Orthoclone and OKTcdr4a (Janssen-Cilag)), anti-CD3 antibodies (*e.g.*, Nuvion (Product Design Labs), OKT3 (Johnson & Johnson), or Rituxan (IDEC)), anti-CD5 antibodies (*e.g.*,

an anti-CD5 ricin-linked immunoconjugate), anti-CD7 antibodies (*e.g.*, CHH-380 (Novartis)), anti-CD8 antibodies (Pharmingen), anti-CD40 ligand monoclonal antibodies (*e.g.*, IDEC-131 (IDEC)), anti-CD52 antibodies (*e.g.*, CAMPATH 1H (Ilex)), anti-CD2 antibodies, anti-CD11a antibodies (*e.g.*, Xanelim (Genentech)), and anti-B7 antibodies (*e.g.*, IDEC-114) (IDEC))), CTLA4-immunoglobulin LFA3TIP (Biogen), cytokines (*e.g.*, interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, TNF-α, TNF-β, interferon (IFN)-α, IFN-β, IFN-γ, and GM-CSF), anti-cytokine receptor antibodies (*e.g.*, anti-IFN receptor antibodies, anti-IL-2 receptor antibodies (*e.g.*, Zenapax (Protein Design Labs)), anti-IL-4 receptor antibodies, anti-IL-6 receptor antibodies, anti-IL-10 receptor antibodies, and anti-IL-12 receptor antibodies), and anti-cytokine antibodies (*e.g.*, anti-IFN antibodies, anti-TNF-α antibodies, anti-IL-1β antibodies, anti-IL-6 antibodies, anti-IL-8 antibodies (*e.g.*, ABX-IL-8 (Abgenix)), anti-IL-9 antibodies, and anti-IL-12 antibodies).

In a preferred embodiment, proteins, polypeptides or peptides (including antibodies) that are utilized as immunomodulatory agents are derived from the same species as the recipient of the proteins, polypeptides or peptides so as to reduce the likelihood of an immune response to those proteins, polypeptides or peptides. In another preferred embodiment, when the subject is a human, the proteins, polypeptides, or peptides that are utilized as immunomodulatory agents are human or humanized.

In accordance with the invention, one or more immunomodulatory agents are administered to a subject with a solid tumor cancer to reduce or inhibit one or more aspects of the immune response. Any technique well-known to one skilled in the art can be used to measure one or more aspects of the immune response in a particular subject, and thereby determine when it is appropriate to administer an immunomodulatory agent in combination with a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria) to said subject in accordance with the invention. In a preferred embodiment, a mean absolute lymphocyte count of approximately 500 cells/mm^3, preferably 600 cells/mm^3, 650 cells/mm^3, 700 cells/mm^3, 750 cells/mm^3, 800 cells/mm^3, 900 cells/mm^3, 1000 cells/mm^3, 1100 cells/mm^3, or 1200 cells/mm^3 is maintained in a subject. In another preferred embodiment, a subject with a solid tumor cancer is not administered an immunomodulatory agent if his/her mean absolute lymphocyte count is 500 cells/mm^3 or less, 550 cells/mm^3 or less, 600 cells/mm^3 or less, 650 cells/mm^3 or less, 700 cells/mm^3 or less, 750 cells/mm^3 or less, 800 cells/mm^3 or less, 900 cells/mm^3 or less or 1000 cells/mm^3 or less.

In accordance with the invention, one or more immunomodulatory agents are administered to a subject with a solid tumor cancer prior to, subsequent to, or

concomitantly with tumor-targeted bacteria of the invention. Preferably, one or more immunomodulatory agents are administered to a subject with a solid tumor cancer to reduce or inhibit one or more aspects of the immune response as necessary. Any technique well-known to one skilled in the art can be used to measure one or more aspects of the immune response in a particular subject, and thereby determine when it is necessary to administer an immunomodulatory agent to said subject. In a preferred embodiment, one or more immunomodulatory agents are administered to a subject with a solid tumor cancer so as to transiently reduce or inhibit one or more aspects of the immune response. Such a transient inhibition or reduction of one or more aspects of the immune system can last for hours, days, weeks, or months. Preferably, the transient inhibition or reduction in one or more aspects of the immune response last for a few hours (*e.g.*, 2 hours, 4 hours, 6 hours, 8 hours, 12 hours, 14 hours, 16 hours, 18 hours, 24 hours, 36 hours, or 48 hours), a few days (*e.g.*, 3 days, 4 days, 5 days, 6 days, 7 days, or 14 days), or a few weeks (*e.g.*, 3 weeks, 4 weeks, 5 weeks or 6 weeks). The transient reduction or inhibition of one or more aspects of the immune response enhances the tumor-targeting capabilities of the tumor-targeted bacteria of the invention.

Nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity or proteins, polypeptides, or peptides with immunomodulatory activity can be administered to a subject with a solid tumor cancer in accordance with the methods of the invention. Further, nucleic acid molecules encoding derivatives, analogs, fragments or variants of proteins, polypeptides, or peptides with immunomodulatory activity, or derivatives, analogs, fragments or variants of proteins, polypeptides, or peptides with immunomodulatory activity can be administered to a subject with a solid tumor cancer in accordance with the methods of the invention. Prefereably, such derivatives, analogs, variants and fragments retain the immunomodulatory activity of the full-length wild-type protein, polypeptide, or peptide.

In one embodiment of the invention, at least one immunomodulatory agent administered to a subject with a solid tumor cancer in accordance with the methods of the invention reduces or depletes T cells in said subject. See, *e.g.*, U.S. Pat. No. 4,658,019. In a preferred embodiment of the invention, at least one immunomodulatory agent administered to a subject with a solid tumor cancer in accordance with the methods of the invention inactivates $CD8^+$ T cells. As a result of such treatment, immune responses to tumor-targeted bacteria administered prior to, concomitantly with, or subsequent to the administration of the immunomodulatory agent are avoided or reduced. In a specific embodiment, anti-CD8 antibodies are used to reduce or deplete $CD8^+$ T cells. Preferably,

human or humanized anti-CD8 antibodies are administered to a human subject to prevent the subject from mounting an immune response to the tumor-targeted bacteria. Depletion of $CD8^+$ T cells has been shown to inhibit CTL-mediated elimination of the bacterial infection.

Antibodies that interfere with or block the interactions necessary for the activation of B cells by TH (T helper) cells, and thus block the production of neutralizing antibodies, are useful as immunomodulatory agents in the methods of the invention. For example, B cell activation by T cells requires certain interactions to occur (Durie et al, Immunol. Today, 15(9):406-410 (1994)), such as the binding of CD40 ligand on the T helper cell to the CD40 antigen on the B cell, and the binding of the CD28 and/or CTLA4 ligands on the T cell to the B7 antigen on the B cell. Without both interactions, the B cell cannot be activated to induce production of the neutralizing antibody.

The CD40 ligand (CD40L)-CD40 interaction is a desirable point to block the immune response to tumor-targeted bacteria because of its broad activity in both T helper cell activation and function as well as the absence of redundancy in its signaling pathway. Thus, in a preferred embodiment of the invention, the interaction of CD40L with CD40 is transiently blocked at the time of administration of one or more tumor-targeted bacteria. This can be accomplished by treating with an agent which blocks the CD40 ligand on the TH cell and interferes with the normal binding of CD40 ligand on the T helper cell with the CD40 antigen on the B cell. Blocking the CD40L-CD40 interaction prevents the activation of the T helper cells that contribute to problems associated with the stability and re-administration of the tumor-targeted bacteria. Thus, an antibody to CD40 ligand (anti-CD40L) (available from Bristol-Myers Squibb Co; see, *e.g.*, European patent application 555,880, published Aug. 18, 1993) or a soluble CD40 molecule can be selected and used as an immunomodulatory agent in accordance with the methods of the invention.

In another embodiment, at least one immunomodulatory agent which reduces or inhibits one or more biological activities (*e.g.*, the differentiation, proliferation, and/or effector functions) of TH0, TH1, and/or TH2 subsets of $CD4^+$ T helper cells is administered to a subject with a solid tumor cancer in accordance with the methods of the invention. One example of such an immunomodulatory agent is IL-4. IL-4 enhances antigen-specific activity of TH2 cells at the expense of the TH1 cell function (see, *e.g.*, Yokota et al, 1986 Proc. Natl. Acad. Sci., USA, 83:5894-5898; and U.S. Pat. No. 5,017,691). Other examples of immunomodulatory agents that affect the biological activity (*e.g.*, proliferation, differentiation, and/or effector functions) of T-helper cells (in particular, TH1 and/or TH2 cells) include, but are not limited to, IL-6, IL-10, IL-12, and interferon (IFN)-γ.

In another embodiment, at least one immunomodulatory agent administered to a subject with a solid tumor cancer in accordance with the methods of the invention is a cytokine that prevents antigen presentation. In a preferred embodiment, at least one immunomodulatory agent used in the methods of the invention is IL-10. IL-10 also reduces or inhibits macrophage action which involves bacterial elimination.

Other examples of immunomodulatory agents which can be used in accordance with the invention include, but are not limited to, corticosteroids, azathioprine, mycophenolate mofetil, cyclosporin A, hydrocortisone, FK506, methotrexate, leflunomide, and cyclophosphamide. A short course of cyclophosphamide has been demonstrated to successfully interrupt both $CD4^+$ and $CD8^+$ T cell activation to adenoviral capsid protein (Jooss et al., 1996, Hum. Gene Ther. 7:1555-1566), and at higher doses, formation of neutralizing antibody was prevented. Hydrocortisone or cyclosporin A treatment has been successfully used to decrease the induction of cytokines, some of which may be involved in the clearance of bacterial infections.

Proteins, polypeptides, or peptides that can be used as immunomodulatory agents can be produced by any technique well-known in the art. See, *e.g.*, Chapter 16 Ausubel et al. (eds.), 1999, Short Protocols in Molecular Biology, Fourth Edition, John Wiley & Sons, NY, which describes methods of producing proteins, polypeptides, or peptides, and which is incorporated herein by reference in its entirety. Antibodies which can be used as immunomodulatory agents can be produced by, *e.g.*, methods described in U.S. Patent No. 6,245,527 and in Harlow and Lane Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988, which are incorporated herein by reference in their entirety. Preferably, agents that are commercially available and known to function as immunomoulatory agents are used in the compositions and methods of the invention. The immunomodulatory activity of an agent can be determined *in vitro* and/or *in vivo* by any technique well-known to one skilled in the art, including, *e.g.*, by CTL assays, proliferation assays, and immunoassays (*e.g.* ELISAs) for the expression of particular proteins such as co-stimulatory molecules and cytokines.

5.2. TUMOR-TARGETED BACTERIA

Any tumor-targeted bacteria may be used in the methods and compositions of the invention. In a specific embodiment, the tumor-targeted bacteria used in the methods of the invention are facultative aerobes or facultative anaerobes. Examples of tumor-targeted bacteria that are facultative aerobes or facultative anaerobes which may be used in the methods of the invention include, but are not limited to, *Escherichia coli* including

enteroinvasive *Escherichia coli, Salmonella spp., Shigella spp., Yersinia enterocohtica, Listeria monocytogenies, Mycoplasma hominis,* and *Streptococcus spp.*. In a preferred embodiment, the facultative aerobes or facultative anaerobes used in the methods and compositions for the invention are attenuated. In another specific embodiment, the tumor-targeted bacteria used in the methods of the invention are obligate anaerobes. Examples of obligate anaerobes include, but are not limited to, *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp., *Peptostreptococcus* sp., and any spores thereof. See, *e.g.*, the following references for a review of such bacteria: Bartlett, J. G. 1990. Anaerobic Gram-Positive Nonsporulating Bacteria. Chapter 225, pp 1869-1870, In: Mandell, G. L., Douglas, R. G. and Bennett, J. E. (Eds.) Principles and Practice of Infectious Diseases, Third Edition. Churchill Livingstone, NY 2340 pp and 106 pp Index; Bartlett *Id.* Chapter 219, pp 1828-1842, Bartlett Id. Chapter 222, pp 1850-1860;Bartlett Id. Chapter 224, pp 1867-1869; Lerner, P. I. 1990. Actinomyces and Arachnia Species. Chapter 233, pp 1932-1942. In: Mandell, G. L., Douglas, R. G. and Bennett, J. E. (Eds.) Principles and Practice of Infectious Diseases, Third Edition. Churchill Livingstone, NY 2340 pp and 106 pp Index; and Lerner, P. I. 1990.. Nocardia Species. Chapter 232, pp 1926-1932. In: Mandell, G. L., Douglas, R. G. and Bennett, J. E. (Eds.) Principles and Practice of Infectious Diseases, Third Edition. Churchill Livingstone, NY 2340 pp and 106 pp Index. In a preferred embodiment, the obligate anaerobes used in the methods and compositions of the invention are attenuated or non-pathogenic. In certain embodiments, the tumor-targeted bacteria used in the methods and compositions of the invention are not obligate anaerobes. In other embodiments, the tumor-targeted bacteria used in the methods and compositions of the invention is not a *Clostridium* sp.. In yet other embodiments, the tumor-targeted bacteria used in the methods and compositions of the invention is not *Bifidobacterium* sp..

In a preferred embodiment, the tumor-targeted bacteria used in the methods and compositions of the invention are attenuated. Any technique well-known to one of skill in the art may be used to attenuate the tumor-targeted bacteria. Preferably, the gram-negative tumor-targeted bacteria used in the methods and compositions of the invention are attenuated by introducing one or more mutations in one or more genes in the lipopolysaccharide (LPS) biosynthetic pathway, and optionally one or more mutations to auxotrophy for one or more nutrients or metabolites. The attenuated tumor-targeted bacteria induce lower levels of tumor necrosis factor-α (TNF-α) than their wild-type counterpart (*i.e.*, about 5% to about 40%, about 5% to about 35%, about 5% to about 25%, about 5% to about 15%, or about 5% to about 10% of TNF-α induced by wild-type), and

thus, avoid or reduce the risk of inducing septic shock in a subject with a solid tumor cancer when administered to said subject in accordance with the methods of the invention.

Factors contributing to attenuation and tumor-targeting may be used to construct or select an appropriate tumor-targeted bacteria for use in the methods and compositions of the invention. For example, methods to select and isolate tumor-targeted bacteria are described in Section 6.1, and methods to attenuate bacteria are described in Section 6.2 of International Publication No. WO 96/40238, which are incorporated herein by reference. Examples of attenuated tumor-targeted bacteria are also described in International Publication No. WO 99/13053, which is incorporated herein by reference in its entirety.

In one embodiment of the invention, the tumor-targeted bacteria are themselves therapeutically active against a solid tumor cancer. Said tumor-targeted bacteria can inhibit or reduce the growth of a tumor or tumor cells, reduce the volume of a tumor, kill tumor cells, inhibit or reduce the spread of tumor cells (metastasis), or ameliorate one or more symptoms associated with a cancer. In particular, said tumor-targeted bacteria can kill, suppress the growth of, suppress the division of, or induce apoptosis of tumor cells. Further, said tumor-targeted bacteria can affect tumor cells by directly infecting and/or lysing said cells, or by releasing factors which kill, prevent the growth of, prevent the division of, or induce apoptosis of the tumor cells.

The present invention encompasses the use of tumor-targeted bacteria which replicate at physiological temperatures and inhibit or reduce tumor cell growth *in vitro* or *in vivo* in the compositions and methods of the invention. Preferably, such tumor-targeted bacteria inhibit or reduce tumor cell growth *in vivo*. The present invention also encompasses the use of tumor-targeted bacteria which replicate at physiological temperatures but do not inhibit or reduce tumor cell growth or *in vivo* in the compositions and methods of the invention. In accordance with the invention, such tumor-targeted bacteria may be engineered to contain or express one or more therapeutic molecules. In one embodiment, a tumor-targeted bacteria which replicates at physiological temperatures in a solid tumor but does not inhibit or reduce tumor cell growth is a derivative of a tumor-targeted bacteria which replicates at physiological temperatures and inhibits or reduces tumor cell growth. In accordance with this embodiment, such derivatives may be obtained by growing a tumor-targeted bacteria which replicates at physiological temperatures and inhibits or reduces tumor cell growth under various selective pressures or by random mutagenesis (*e.g.*, by exposing the bacteria to various mutagens). In another embodiment, a tumor-targeted bacteria which replicates at physiological temperatures and inhibits or reduces tumor cell growth is a derivative of a tumor-targeted bacteria which

replicates at physiological temperatures in a solid tumor but does not inhibit or reduce tumor cell growth. In accordance with this embodiment, such derivatives may be obtained by growing a tumor-targeted bacteria which replicates at physiological temperatures but does not inhibit or reduce tumor cell growth under various selective pressures or by random mutagenesis (*e.g.*, by exposing the bacteria to various mutagens). Examples of growth condition parameters which may be varied include, but are not limited to, temperature, type of media, and pH. Examples of mutagens which may be used include, but are not limited to, ultraviolet light and nitrosoguanadine. Derivatives of attenuated tumor-targeted bacteria and methods of producing such derivatives are described in Section 6.1 of International Publication No. WO 99/13053.

While the teachings in sections of this application refer specifically to *Salmonella*, the compositions and methods of the invention are in no way meant to be restricted to *Salmonella* but encompass any other bacterium to which the teachings apply. Suitable bacteria include, but are not limited to, *Escherichia coli* including enteroinvasive *Escherichia coli, Salmonella spp., Shigella spp., Yersinia enterocohtica, Listeria monocytogenies, Mycoplasma hominis, Streptococcus spp.*, wherein the bacterium is a facultative aerobe or facultative anaerobe. Suitable bacteria also include obligate anaerobes such as, *e.g.*, *Clostridium* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Propionibacterium* sp., *Peptostreptococcus* sp., and any spores thereof.

5.2.1. *SALMONELLA*

The present invention encompasses the use of tumor-targeted *Salmonella* in the methods and compositions of the invention. In a preferred embodiment, the tumor-targeted *Salmonella* used in the methods and compositions of the invention are attenuated. Suitable methods for obtaining attenuated tumor-targeted *Salmonella* mutants are described in Section 6.1 (pages 25-32; tumor-targeting) and Section 6.2.2 (pages 43-51; attenuation) of International Publication No. WO 96/40238, which are incorporated herein by reference. Preferably, the attenuated tumor-targeted *Salmonella* mutants used in the methods and compositions of the invention have one or more mutations in one or more genes which reduce the virulence and toxicity of *Salmonella*. In a preferred embodiment, the attenuated tumor-targeted *Salmonella* mutants used in the methods and compositions of the invention have mutation(s) in one or more genes in the lipopolysaccharide (LPS) biosynthetic pathway (preferably in the *msbB* gene) and optionally, have one or more mutations to auxotrophy for one or more nutrients or metabolites, such as uracil biosynthesis, purine biosynthesis, and arginine biosynthesis.

An illustrative example of an attenuated tumor-targeted *Salmonella* mutant having an LPS pathway mutant is the *msbB- Salmonella* mutant described in International Publication No. WO 99/13053, which is incorporated herein by reference in its entirety; see especially Section 6.1.2 which describes the characteristic of the *msbB- Salmonella* mutant. One characteristic of the *msbB- Salmonella* mutant is its decreased ability to induce a TNF-α response compared to the wild-type bacteria. In certain embodiments, the *msbB- Salmonella* mutant induces TNF-α expression at levels of about 5 percent to about 40 percent or about 5 percent to about 30 percent, about 5 percent to about 20 percent, or about 5 percent to about 10 percent compared to the levels induced by wild-type *Salmonella*, as measured by techniques well-known in the art including, *e.g.*, immunoassays such as ELISAs.

The growth of an attenuated tumor-targeted *Salmonella* used in accordance with the invention may be sensitive to a chelating agent such as, *e.g.*, Ethylenediaminetetraacetic Acid (EDTA), Ethylene Glycol-bis(β-aminoethyl Ether) N, N, N', N'-Tetraacetic Acid (EGTA), or sodium citrate. For example, a chelating agent may inhibit the growth of an attenuated tumor-targeted *Salmonella* by about 90%, 95%, 99%, or 99.5% compared to the growth of a wild-type *Salmonella* sp. Preferably, the attenuated tumor-targeted *Salmonella* used in accordance with the invention survive in macrophages at about 50% to about 30%, about 30% to about 10%, or about 10% to about 1% of the level of survival of a wild-type *Salmonella* sp.

The present invention encompasses the use of tumor-targeted *Salmonella* mutants which replicate at physiological temperatures and inhibit or reduce tumor cell growth *in vitro* or *in vivo* in the compositions and methods of the invention. Preferably, such tumor-targeted *Salmonella* mutants inhibit or reduce tumor cell growth *in vivo*. The present invention also encompasses the use of tumor-targeted *Salmonella* mutants which replicate at physiological temperatures but do not inhibit or reduce tumor cell growth *in vivo* in the compositions and methods of the invention. In accordance with the invention, such tumor-targeted *Salmonella* mutants may be engineered to contain or express one or more therapeutic molecules for use in the methods and compositions of the invention. In one embodiment, tumor-targeted *Salmonella* mutants which replicate at physiological temperatures in a solid tumor but do not inhibit or reduce tumor cell growth are derivatives of tumor-targeted *Salmonella* mutants which replicate at physiological temperatures and inhibit or reduce tumor cell growth. In accordance with this embodiment, such derivatives may be obtained by growing tumor-targeted *Salmonella* mutants which replicate at physiological temperatures and inhibit or reduce tumor cell growth under various selective

pressures or by randomly mutagenizing the bacteria (by, *e.g.*, exposing the bacteria to various mutagens). In another embodiment, tumor-targeted *Salmonella* mutants which replicate at physiological temperatures and inhibit or reduce tumor cell growth are derivatives of tumor-targeted *Salmonella* mutants which replicate at physiological temperatures in a solid tumor but do not inhibit or reduce tumor cell growth. In accordance with this embodiment, such derivatives may be obtained by growing tumor-targeted *Salmonella* mutants which replicate at physiological temperatures but do not inhibit or reduce tumor cell growth under various selective pressures or by randomly mutagenizing the bacteria (by, *e.g.*, exposing the bacteria to one or more of various mutagens). Derivatives of tumor-targeted *Salmonella* mutants and methods of producing such derivatives are described in Sections 6.1, 7.7 and 7.9 of International Publication No. WO 99/13053, which is incorporated herein by reference in its entirety.

In a specific embodiment, the attenuated tumor-targeted *Salmonella* utilized in accordance with the invention is the strain designated YS1456 (a.k.a., 8.7) deposited with the ATCC and assigned Accession No. 202164. In a preferred embodiment, the attenuated tumor-targeted *Salmonella* utilized in accordance with the invention is the strain designated VNP20009 (a.k.a., YS1646 or 41.2.9) deposited with the ATCC and assigned Accession No. 202165. The strains YS1456 and VNP20009 are described in International Publication Nos. WO 99/13053 and WO 01/25397, in particular Section 6 of International Publication No. WO 99/13053.

5.3. THERAPEUTIC MOLECULES

The tumor-targeted bacteria used in the methods and compositions of the invention may constitutively express or be induced to express one or more therapeutic molecules. In certain embodiments, the tumor-targeted bacteria naturally encodes for and constitutively or inducibly expresses one or more therapeutic molecules. In certain other embodiments, the tumor-targeted bacteria is engineered to constitutively or inducibly express one or more therapeutic molecules.

Therapeutic molecules include, but are not limited to, proteinaceous molecules, including, but not limited to, peptide, polypeptide, protein, post-translationally modified protein, antibodies etc., or nucleic acid molecules including, but not limited to, double-stranded or single-stranded DNA, or double-stranded or single-stranded RNA, as well as triple helix nucleic acid molecules. The nucleic acid molecule can function as a ribozyme, or antisense nucleic acid, etc.

A therapeutic molecule of the invention can be the product of an enzymatic reaction. The tumor-targeted bacteria can naturally express or be engineered to express said enzyme. The therapeutic molecule which is a product of such an enzymatic reaction can be a metabolite, a small molecule, an inorganic, or an organic compound. The therapeutic molecule which is a product of an enzymatic reaction can inhibit or reduce the growth of a tumor or tumor cells, reduce the volume of a tumor, kill tumor cells, inhibit or reduce the spread of tumor cells (metastasis), or ameliorate one or more symptoms associated with a cancer. On a cellular level, the therapeutic molecule which is a product of an enzymatic reaction can kill, suppress the growth of, suppress the division of, or induce apoptosis of the cells of the tumor.

The therapeutic molecules can be derived from any known organism, including, but not limited to, animals, plants, bacteria, fungi, and protista, or viruses. In a preferred embodiment of the invention, the therapeutic molecule is derived from a mammal. In a more preferred embodiment, the therapeutic molecule is derived from a human. Examples of therapeutic molecules include, but are not limited to, anti-angiogenic factors, cytotoxic factors, cytostatic factors, pro-apoptotic factors, tumor inhibitory enzymes, and functional fragments thereof.

In a specific embodiment, the therapeutic molecules of the invention are members of the TNF family or functional fragments thereof. Examples of TNF family members, include, but are not limited to, tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), TNF-α-related apoptosis-inducing ligand (TRAIL), TNF-α-related activation-induced cytokine (TRANCE), TNF-α-related weak inducer of apoptosis (TWEAK), CD40 ligand (CD40L), LT-α, LT-β, OX4OL, CD4OL, FasL, CD27L, CD30L, 4-1BBL, APRIL, LIGHT, TL1, TNFSF16, TNFSF17, and AITR-L. In a preferred embodiment, a therapeutic molecule of the invention is tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), TNF-α-related apoptosis-inducing ligand (TRAIL), TNF-α-related activation-induced cytokine (TRANCE), TNF-α-related weak inducer of apoptosis (TWEAK), CD40 ligand (CD40L), or a functional fragment thereof. For review see, *e.g.*, Kwon, B. *et al.*, 1999, Curr. Opin. Immunol. 11:340-345, which describes members of the TNF family. Also, Table 1 herein below, lists classic and standardized nomenclature of exemplary members of the TNF family.

TABLE 1

TNF FAMILY MEMBERS	
Classic Nomencalture	Standardized Nomenclature
LT-α	TNFSF1
TNF-α	TNFSF2
LT-β	TNFSF3
OX4OL	TNFSF4
CD4OL	TNFSF5
$F_{as}L$	TNFSF6
CD27L	TNFSF7
CD30L	TNFSF8
4-1BBL	TNFSF9
TRAIL	TNFSF10
TRANCE	TNFSF11
TWEAK	TNFSF12
APRIL	TNFSF13
LIGHT	TNFSF14
TL1	TNFSF15
---	TNFSF16
---	TNFSF17
AITR-L	TNFSF18

In another specific embodiment, the therapeutic molecules of the invention are anti-angiogenic factors or functional fragments thereof. Examples of anti-angiogenic factors, include, but are not limited to, endostatin, angiostatin, apomigren, anti-angiogenic antithrombin III, the 29 kDa N–terminal and a 40 kDa C-terminal proteolytic fragments of fibronectin, a uPA receptor antagonist, the 16 kDa proteolytic fragment of prolactin, the 7.8 kDa proteolytic fragment of platelet factor-4, the anti-angiogenic 24 amino acid fragment of platelet factor-4, the anti-angiogenic factor designated 13.40, the anti-angiogenic 22 amino acid peptide fragment of thrombospondin I, the anti-angiogenic 20 amino acid peptide fragment of SPARC, RGD and NGR containing peptides, the small anti-angiogenic

peptides of laminin, fibronectin, procollagen and EGF, peptide antagonists of integrin $\alpha_v\beta_3$ and the VEGF receptor (VEGF), anti-VEGFR antibodies, and anti-integrin $\alpha_v\beta_3$.

In a preferred embodiment of the invention, a therapeutic molecule of the invention is endostatin. Naturally occurring endostatin consists of the C-terminal ~180 amino acids of collagen XVIII (cDNAs encoding two splice forms of collagen XVIII have GenBank Accession Nos. AF18081 and AF18082).

In another preferred embodiment of the invention, a therapeutic molecule of the invention is a plasminogen fragment (the coding sequence for plasminogen can be found in GenBank Accession Nos. NM_000301 and A33096). Angiostatin peptides naturally include the four kringle domains of plasminogen, kringle 1 through kringle 4. It has been demonstrated that recombinant kringle 1, 2 and 3 possess the anti-angiogenic properties of the native peptide, whereas kringle 4 has no such activity (Cao et al., 1996, J. Biol. Chem. 271:29461-29467). Accordingly, the angiostatin therapeutic molecule of the invention comprises at least one and preferably more than one kringle domain selected from the group consisting of kringle 1, kringle 2 and kringle 3. In a specific embodiment, the therapeutic molecule of the invention is the 40 kDa isoform of the human angiostatin molecule, the 42 kDa isoform of the human angiostatin molecule, the 45 kDa isoform of the human angiostatin molecule, or a combination thereof. In another embodiment, the therapeutic molecule is the kringle 5 domain of plasminogen, which is a more potent inhibitor of angiogenesis than angiostatin (angiostatin comprises kringle domains 1-4).

In another preferred embodiment of the invention, a therapeutic molecule of the invention is antithrombin III. Antithrombin III, which is referred to hereinafter as antithrombin, comprises a heparin binding domain that tethers the protein to the vasculature walls, and an active site loop which interacts with thrombin. When antithrombin is tethered to heparin, the protein elicits a conformational change that allows the active loop to interact with thrombin, resulting in the proteolytic cleavage of said loop by thrombin. The proteolytic cleavage event results in another change of conformation of antithrombin, which (i) alters the interaction interface between thrombin and antithrombin and (ii) releases the complex from heparin (Carrell, 1999, Science 285:1861-1862, and references therein). O'Reilly *et al.* (1999, Science 285:1926-1928) have discovered that the cleaved antithrombin has potent anti-angiogenic activity. Accordingly, in one embodiment, the anti-angiogenic factor of the invention is the anti-angiogenic form of antithrombin. For the delivery of said protein to a solid tumor according to the methods of the invention, the tumor-targeted bacteria is modified to express full length antithrombin GenBank Accession No. NM_000488 and a proteolytic enzyme that catalyzes the cleavage of antithrombin to

produce the anti-angiogenic form of the protein. The proteolytic enzyme is selected from the group comprising thrombin, pancreatic elastases, and human neutrophil elastase. In a preferred embodiment, the proteolytic enzyme is pancreatic elastase. Methods for the recombinant expression of functional pancreatic elastase are taught by Shirasu (Shirasu *et al.*, 1987, J. Biochem. 102:1555-1563).

In another preferred embodiment of the invention, a therapeutic molecule of the invention is the 40 kDa and/or 29 kDa proteolytic fragment of fibronectin. The expression vehicles for these fragments can be generated by standard methods using the full length nucleic acid sequence encoding the fibronectin precursor protein (GenBank Accession No. X02761), and a description of the maturation of the encoded protein. In a preferred embodiment, the 40 kDa and/or the 29 kDa fragment of fibronectin is expressed as a cytoplasmic protein under the control of the *trc* promoter, for example by insertion into the pTrc99A plasmid.

In another preferred embodiment of the invention, a therapeutic molecule of the invention is a urokinase plasminogen activator (uPA) receptor antagonist. In one mode of the embodiment, the antagonist is a dominant negative mutant of uPA (see, *e.g.*, Crowley *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:5021-5025). In another mode of the embodiment, the antagonist is a peptide antagonist or a fusion protein thereof (Goodson *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:7129-7133). In yet another mode of the embodiment, the antagonist is a dominant negative soluble uPA receptor (Min et al., 1996, Cancer Res. 56:2428-2433).

In a preferred embodiment of the invention, a therapeutic molecule of the invention is the 16 kDa N-terminal fragment of prolactin, comprising approximately 120 amino acids, or a biologically active fragment thereof (the coding sequence for prolactin can be found in GenBank Accession No. NM_000948). In a specific embodiment, said prolactin fragment has a Cys58 →Ser58 mutation to circumvent undesired cross-linking of the protein by disulfide bonds.

In another preferred embodiment of the invention, a therapeutic molecule of the invention is the 7.8 kDa platelet factor-4 fragment. In a specific embodiment, the 7.8 kDa platelet factor-4 fragment is expressed as a fusion protein wherein the amino terminal comprises the first 35 amino acids of *E. coli* β-glucoronidase. In another embodiment, the heparin binding lysines of platelet factor-4 are mutated to glutamic acid residues, which results in a variant protein having potent anti-angiogenic activity (Maione *et al.*, 1991, Cancer Res. 51:2077-2083). The coding sequence for platelet factor-4 has the GenBank Accession No. NM_002619.

In another preferred embodiment of the invention, a therapeutic molecule of the invention is a small peptide corresponding to the anti-angiogenic 13 amino acid fragment of platelet factor-4, the anti-angiogenic factor designated 13.40, the anti-angiogenic 22 amino acid peptide fragment of thrombospondin I , the anti-angiogenic 20 amino acid peptide fragment of SPARC, the small anti-angiogenic peptides of laminin, fibronectin, procollagen, or EGF, or small peptide antagonists of integrin $\alpha_v\beta_3$ or the VEGF receptor. In a specific embodiment, the small peptides are expressed in tandem to increase protein stability. The sequences of the small peptides are provided by Cao (1998, Prog. Mol. Subcell. Biol. 20:161-176), with the exception of VEGF receptor antagonists (Soker *et al.*, 1993, J. Biol. Chem. 272:31582-31588). In a highly preferred embodiment, the small peptide comprises an RGD or NGR motif. In certain modes of the embodiment, the RGD or NGR containing peptide is presented on the cell surface of the host bacteria, for example, by fusing the nucleic acid encoding the peptide in frame with a nucleic acid encoding one or more extracellular loops of OmpA.

In another specific embodiment, the therapeutic molecules of the invention are cytotoxic polypeptides or peptides, or functional fragments thereof. A cytotoxic polypeptide or peptide is cytotoxic or cytostatic to a cell, for example, by inhibiting cell growth through interference with protein synthesis or through disruption of the cell cycle. Such a product may act by cleaving rRNA or ribonucleoprotein, inhibiting an elongation factor, cleaving mRNA, or other mechanism that reduced protein synthesis to a level such that the cell cannot survive.

Examples of cytotoxic polypeptides or peptides include, but are not limited to, members of the bacteriocin family, verotoxin, cytotoxic necrotic factor 1 (CNF1; *e.g.*, *E. coli* CNF1 and *Vibrio fischeri* CNF1), cytotoxic necrotic factor 2 (CNF2), *Pasteurella multiocida* toxin (PMT), hemolysin, CAAX tetrapeptides which are potent competitive inhibitors of farnesyltransferase, saporin, the ricins, abrin, other ribosome inactivating proteins (RIPs), *Pseudomonas* exotoxin, inhibitors of DNA, RNA or protein synthesis, antisense nucleic acids, other metabolic inhibitors (*e.g.*, DNA or RNA cleaving molecules such as DNase and ribonuclease, protease, lipase, phospholipase), prodrug converting enzymes (*e.g.*, thymidine kinase from HSV and bacterial cytosine deaminase), light-activated porphyrin, ricin, ricin A chain, maize RIP, gelonin, cytolethal distending toxin, diphtheria toxin, diphtheria toxin A chain, trichosanthin, tritin, pokeweed antiviral protein (PAP), mirabilis antiviral protein (MAP), Dianthins 32 and 30, abrin, monodrin, bryodin, shiga, a catalytic inhibitor of protein biosynthesis from cucumber seeds (see, *e.g.*, International Publication WO 93/24620), *Pseudomonas* exotoxin, *E. coli* heat-labile toxin,

E. coli heat-stable toxin, EaggEC stable toxin-1 (EAST), biologically active fragments of cytotoxins, enterotoxins, and others known to those of skill in the art. See, *e.g.*, O'Brian and Holmes, Protein Toxins of *Escherichia coli* and *Salmonella* in Escherichia and Salmonella, Cellular and Molecular Biology, Neidhardt et al. (eds.), pp. 2788-2802, ASM Press, Washington, D.C. for a review of *E. coli* and *Salmonella* toxins.

In certain embodiments, the therapeutic molecule of the invention is cytotoxic or cytostatic to a cell by inhibiting cell growth through interference with protein synthesis or through disruption of the cell cycle. Such a product may act, for example, by cleaving rRNA or ribonucleoprotein, inhibiting an elongation factor, cleaving mRNA, or another mechanism that reduces protein synthesis to a level such that the cell cannot survive. Examples of such therapeutic molecules include, but are not limited to, saporin, the ricins, abrin, and other ribosome inactivating proteins (RIPs).

In another embodiment, the therapeutic molecule of the invention is a pro-drug converting enzyme, *i.e.*, an enzyme that modulates the chemical nature of a drug to produce a cytotoxic agent. Illustrative examples of pro-drug converting enzymes are listed on page 33 and in Table 2 of International Publication No. WO 96/40238 by Pawelek *et al.*, which is incorporated herein by reference. International Publication No. WO 96/40238 also teaches methods for production of secreted fusion proteins comprising such pro-drug converting enzymes. According to the present invention, a pro-drug converting enzyme need not be a secreted protein if co-expressed with a release factor such as BRP. In a specific embodiment, the pro-drug converting enzyme is cytochrome p450 NADPH oxidoreductase which acts upon mitomycin C and porfiromycin (Murray et al., 1994, J. Pharmacol. Exp. Therapeut. 270:645-649). In another embodiment, the therapeutic molecule is co-expressed with a release factor such as BRP, and causes the release of co-factors (*e.g.*, NADH, NADPH, ATP, etc.) which enhance pro-drug converting enzyme activity. In another mode of the embodiment, a therapeutic molecule is co-expressed with a release factor such as BRP, leading to the release of an activated drug (*e.g.*, a drug which is activated within the bacterial cytoplasm or periplasm, and then released from the tumor-targeted bacteria).

In another embodiment, the therapeutic molecule of the invention is an inhibitor of inducible nitric oxide synthase (iNOS) or of endothelial nitic oxide synthase. Nitric oxide (NO) is implicated to be involved in the regulation of vascular growth and in arterosclerosis. NO is formed from L-arginine by nitric oxide synthase (NOS) and modulates immune responses (*e.g.*, inflammatory responses) and cardiovascular responses.

In another embodiment, the therapeutic molecule of the invention is cytotoxic or cytostatic to a cell by inhibiting the production or activity of a protein involved in cell proliferation, such as an oncogene or growth factor (*e.g.*, bFGF, int-2, hst-1/K-FGF, FGF-5, hst-2/FGF-6, FGF-8), or cellular receptor or ligand. The inhibition can be at the level of transcription or translation (mediated by a therapeutic molecule that is a ribozyme or triplex DNA), or at the level of protein activity (mediated by a therapeutic molecule that is an inhibitor of a growth factor pathway, such as a dominant negative mutant).

In another embodiment, the therapeutic molecule of the invention is a cytokine, chemokine, or an immunomodulating protein, such as IL-1, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, endothelial monocyte activating protein-2 (EMAP2), GM-CSF, IFN-γ, IFN-α, MIP-3α, SLC, MIP-3β, or an MHC gene, such as HLA-B7. Delivery of such immunomodulating effector molecules will modulate the immune system, increasing the potential for host antitumor immunity. Alternatively, nucleic acid molecules encoding costimulatory molecules, such as B7.1 and B7.2, ligands for both CD28 and CTLA-4, can also be delivered to enhance T cell mediated immunity. Yet another immunomodulating agent is α-1,3-galactosyl transferase whose expression on tumor cells allows complement-mediated cell killing. Moreover, certain antibodies can modulate the activity of different aspects of the immune system. Such antibodies include, but are not limited to, antibodies specific to CD3 and antibodies specific to the CD40 cell surface molecule. Further, another immunomodulating agent is a tumor-associated antigen, *i.e.* a molecule that is specifically expressed by a tumor cell and not in the non-cancerous counterpart cell, or is expressed in the tumor cell at a higher level than in the non-cancerous counterpart cell. Illustrative examples of tumor-associated antigens are described in Kuby, *Immunology*, W.H. Freeman and Company, New York, NY, 1st Edition (1992), pp. 515-520 which is incorporated by reference herein. Other examples of tumor-associated antigens are known to those of skill in the art. Preferably, the expression of a nucleic acid molecule encoding protein, polypeptide, or peptide with immunomodulatory activity is regulated by an inducible promoter. In certain embodiments of the invention, the therapeutic molecules of the invention are not immunomodulatory agents.

In another embodiment of the invention, the therapeutic molecule of the invention is a Flt-3 ligand.

In a preferred embodiment, the therapeutic molecule of the invention is a member of the bacteroicin family (see *e.g.*, Konisky, 1982, Ann. Rev. Microbiol. 36:125-144). In certain embodiments of the invention, the bacteriocin family member is not a bacteriocin release protein (BRP). In certain other embodiments, the therapeutic molecule is BRP.

Examples of bacteriocin family members, include, but are not limited to, ColE1, ColE1a, ColE1b ColE2, ColE3, ColE4, ColE5, ColE6, ColE7, ColE8, ColE9, Colicin A, Colicin K, Colicin L, Colicin M, cloacin DF13, pesticin A1122, staphylococcin 1580, butyricin 7423, pyocin R1 or AP41, megacin A-216, microcin M15, and vibriocin (Jayawardene and Farkas-Himsley,1970, J. Bacteriology vol. 102 pp 382-388). Most preferably, the therapeutic molecule is colicin E3 or V, although colicins A, E1, E2, Ia, Ib, K, L, and M (see, Konisky, 1982, Ann. Rev. Microbiol. 36:125-144) are also suitable as a therapeutic molecule. In another preferred mode of this embodiment, the bacteriocin is a cloacin, most preferably cloacin DF13.

In a preferred embodiment, the therapeutic molecule of the invention is ColE1, ColE2, ColE3, ColE4, ColE5, ColE6, ColE7, ColE8, or ColE9. Colicin E3 (ColE3) has been shown to have a profound cytotoxic effect on mammalian cells (Smarda *et al.*, 1978, Folia Microbiol. 23:272-277), including a leukemia cell model system (Fiska *et al.*, 1978, Experientia 35:406-40. ColE3 cytotoxicity is a function of protein synthesis arrest, mediated by inhibition of 80S ribosomes (Turnowsky *et al.*, 1973, Biochem. Biophys. Res. Comm. 52:327-334). More specifically, ColE3 has ribonuclease activity (Saunders, 1978, Nature 274:113-114). In its naturally occurring form, ColE3 is a 60kDa protein complex consisting of a 50kDa and a 10kDa protein in a 1:1 ratio, the larger subunit having the nuclease activity and the smaller subunit having inhibitory function of the 50kDa subunit. Thus, the 50kDa protein acts as a cytotoxic protein (or toxin), and the 10kDa protein acts as an anti-toxin. Accordingly, in one embodiment, when ColE3 is used as a therapeutic molecule, the larger ColE3 subunit or an active fragment thereof is expressed alone or at higher levels than the smaller subunit. In another embodiment of the invention, the ColE3 50kDa toxin and 10kDa anti-toxin are encoded on a single plasmid within tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, such as *Salmonella*. In this embodiment, the toxin/anti-toxin can act as a selection system for the *Salmonella* which carry the plasmid, such that *Salmonella* which lose the plasmid are killed by the toxin. In another embodiment, the 10 kDa anti-toxin is on the chromosome, separate form the colE3 toxin on the plasmid, resulting in a barrier to transmission to other bacteria..

In another preferred embodiment, the therapeutic molecule of the invention is cloacin DF13. Cloacin DF13 functions in an analogous manner to ColE3. The protein complex is of 67KDa molecular weight. The individual components are 57kDa and 9kDa in size. In addition to its ribonuclease activity, DF13 can cause the leakage of cellular potassium.

In another preferred embodiment, the therapeutic molecule of the invention is colicin V (Pugsley, A.P. and Oudega, B. "Methods for Studying Colicins and Their Plasmids" in Plasmids, a Practical Approach 1987, ed. By K.G. Hardy; Gilson, L. *et al.* EMBO J. 9: 3875-3884).

In another embodiment, the therapeutic molecule of the invention is colicin E2 (a dual subunit colicin similar to ColE3 in structure but with endonuclease rather than ribonuclease activity); colicin A, E1, Ia, Ib, or K, which form ion-permeable channels, causing a collapse of the membrane potential of the cell and leading to cell death; colicin L which inhibits protein, DNA & RNA synthesis; colicin M which causes cell sepsis by altering the osmotic environment of the cell; pesticin A1122 which functions in a manner similar to colicin B function; staphycoccin 1580, a pore-forming bacteriocin; butyricin 7423 which indirectly inhibits RNA, DNA and protein synthesis through an unknown target; Pyocin P1, or a protein resembling a bacteriophage tail protein that kills cells by uncoupling respiration from solute transport; Pyocin AP41 which has a colicin E2-like mode of action; megacin A-216 which is a phospholipase that causes leakage of intracellular material (for a general review of bacteriocins, see Konisky, 1982, Ann. Rev. Microbiol. 36:125-144); or colicin A (Pugsley, A.P. and Oudega, B. "Methods for Studying Colicins and Their Plasmids" in Plasmids, a Practical Approach 1987, ed. By K.G. Hardy).

In another specific embodiment, the therapeutic molecules of the invention are tumor inhibitory enzymes or functional fragments thereof. Examples of tumor inhibitory enzymes include, but are not limited, methioninase, asparaginase, lipase, phospholipase, protease, ribonuclease, DNAase, and glycosidase. In a preferred embodiment, the therapeutic molecule is methioninase.

In an embodiment where the therapeutic molecule is a methioninase, examples of a methioninase includes, but is not limited to, L-methionine alpha-deamino-gamma-mercaptomethane-lyase derived from *Pseudomonas*. In one embodiment, the sequence of methioninase is described by Hori *et al.* (1996, Cancer Res. 56(9):2116-22; GenBank Accession No. L43133).

In an embodiment where the therapeutic molecule is an asparaginase, an example of an asparaginase includes, but is not limited to, asparaginase derived from *E. coli*. In a preferred embodiment, the asparaginase derived from *E. coli* has anti-neoplastic activity (see, *e.g.*, GenBank Accession Nos. M34234 and M34277 and Jennings and Beacham, 1990, J. Bact. 172:1491-1498 and Bonthron, 1990, Gene 91:101-105).

In an embodiment where the therapeutic molecule is a phospholipase, an example of a phospholipase includes, but is not limited to, phospholipase C. In a preferred

embodiment, the phospholipase C is isolated from *Salmonella gallinarum* (see, *e.g.*, Singh and Sharma, 1998, Indian J. Exp. Biol. 36:1245-1252). In another embodiment, the phospholipase C is the *Vibrio mimicus* lecithinase (see, *e.g.*, GenBank Accession No. X17300). In yet another embodiment, the phospholipase C is the *Clostridium perfringens* phospholipase C (see, *e.g.*, GenBank Accession No. X17300; Allan et al., 1975, Biochim. Biophys. Acta 413:309-316; and Bunting et al., 1997, J. Clin. Invest. 100:565-574).

In an embodiment where the therapeutic molecule is a protease, an example of a protease includes, but is not limited to a *Serratia marcescens* protease (see, *e.g.*, GenBank Accession No. X04127 and Maeda et al., 1987, Cancer Res. 47:563-566). In a preferred embodiment, the expression of a *Serratia* protease is co-expressed with a protein channel encoded for by, *e.g.*, *prtD, prtE,* or *prtF* protease B secretion genes from *Erwinia chrysanthemi* (see, *e.g.*, Létoffé et al., 1991, J. Bacteriol. 173:2160-2166).

In another embodiment, the therapeutic molecule of the invention is a cytostatic factor. Cytostatic factors inhibit positive regulators of the cell cycle or activate negative regulators of the cell cycle. Delivering cytostatic factors to the solid tumor cancer inhibits or slows down the cell division cycle in cells of the solid tumor cancer, thus inhibiting or reducing the growth of the tumor.

In yet another embodiment, a therapeutic molecule of the invention is a fusion protein (*e.g.*, covalently bonded to a different protein). The invention provides nucleic acids encoding such fusion proteins. In certain other embodiments of this invention, the nucleic acid encoding a fusion protein of the invention is operably linked to an appropriate promoter.

In a specific embodiment, a therapeutic molecule is constructed as a chimeric or fusion protein comprising a therapeutic molecule or fragment thereof (preferably consisting of at least a domain or motif of the effector molecule, or at least 5, at least 10, at least 25, at least 50, at least 75, or at least 100 contiguous amino acids of the therapeutic molecule) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different peptide, polypeptide, or protein. In one embodiment, such a fusion protein or chimeric protein is produced by recombinant expression of a nucleic acid encoding the therapeutic molecule (*e.g.*, a TNF-coding sequence, an anti-angiogenic factor-coding sequence, a tumor inhibitory enzyme-coding sequence, or a cytotoxic polypeptide-coding sequence) joined in-frame to a coding sequence for a different protein. Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product into the expression vehicle of choice by

methods commonly known in the art. Chimeric nucleic acids comprising portions of a nucleic acid encoding a therapeutic molecule fused to any heterologous polypeptide-encoding sequences may be constructed. In a specific embodiment, a chimeric protein comprises a fragment of a therapeutic molecule of at least 5, at least 10, at least 25, at least 50, or at least 100 contiguous amino acids, or a fragment that displays one or more functional activities of the full-length therapeutic molecule.

In a specific embodiment, a fusion protein comprises an affinity tag such as, but not limited to, a hexahistidine tag, or other affinity tag that may be used in purification, isolation, identification, or assay of expression. In another specific embodiment, a fusion protein comprises a protease cleavage site such as a metal protease or serine cleavage site. In this embodiment, it is in some cases preferred that a protease site corresponding to a protease which is active at the site of a tumor is constructed into a fusion protein of the invention. In several embodiments, a therapeutic molecule is constructed as a fusion protein to an Omp-like protein, or fragment thereof (*e.g.*, signal sequence, leader sequence, periplasmic region, transmembrane domain, multiple transmembrane domains, or combinations thereof).

In a preferred embodiment, a therapeutic molecule of the invention is expressed as a fusion protein with an outer membrane protein (Omp-like protein). Bacterial outer membrane proteins are integral membrane proteins of the bacterial outer membrane, possess multiple membrane-spanning domains and are often attached to one or more lipid moieties. Outer membrane proteins are initially expressed in precursor form (the pro-Omp) with an amino terminal signal peptide that directs the protein to the membrane, upon which the signal peptide is cleaved by a signal peptidase to produce the mature protein. In one embodiment, a therapeutic molecule is constructed as a fusion protein with an Omp-like protein. In this embodiment, the therapeutic molecule has enhanced delivery to the outer membrane of the bacteria. Without intending to be limiting as to mechanism, the Omp-like protein is believed by the inventors to act as an anchor or tether for the therapeutic molecule to the outer membrane, or serves to localize the protein to the bacterial outer membrane. In one embodiment, the fusion of a therapeutic molecule to an Omp-like protein is used to enhance localization of a therapeutic molecule to the periplasm. In another embodiment, the fusion of a therapeutic molecule to an Omp-like protein is used to enhance release of a therapeutic molecule. In specific embodiments, the Omp-like protein is at least a portion of OmpA, OmpB, OmpC, OmpD, OmpE, OmpF, OmpT, a porin-like protein, PhoA, PhoE, lamB, β-lactamase, an enterotoxin, protein A, endoglucanase, peptidoglycan-associated lipoprotein (PAL), FepA, FhuA, NmpA, NmpB, NmpC, or a

major outer membrane lipoprotein (such as LPP), etc. In certain embodiments of the invention, the signal sequence is constructed to be more hydrophobic (*e.g.*, by the insertion or replacement of amino acids within the signal sequence to hydrophobic amino acids, *e.g.*, leucine).

In other embodiments of the invention, a fusion protein of the invention comprises a proteolytic cleavage site. The protolytic cleavage site may be endogenous to the therapeutic molecule or endogenous to the Omp-like protein, or the proteolytic cleavage site may be constructed into the fusion protein. In certain specific embodiments, the Omp-like protein of the invention is a hybrid Omp comprising structural elements that originate from separate proteins.

In an exemplary mode of the embodiment, the Omp-like protein is OmpA; the same principles used in the construction of OmpA-like fusion proteins are applied to other Omp fusion proteins, keeping in mind the structural configuration of the specific Omp-like protein.

For example, the native OmpA protein contains eight anti-parallel transmembrane β-strands within the 170 amino acid N-terminal domain of the protein. Between each pair of transmembrane domains is an extracellular or intracellular loop, depending on the direction of insertion of the transmembrane domain. The C-terminal domain consists of 155 amino acids which are located intracellularly and presumably contact the peptidoglycan occupying the periplasmic space. Expression vectors have been generated that facilitate the generation of OmpA fusion proteins. For example, Hobom *et al.* (1995, Dev. Biol. Strand. 84:255-262) have developed vectors containing the OmpA open reading frame with linkers inserted within the sequences encoding the third or fourth extracellular loops that allow the in-frame insertion of the heterologous protein of choice.

In one embodiment of the invention, the portion of the OmpA fusion protein containing the therapeutic molecule has enhanced expression in the periplasm. In one aspect of the embodiment, the fusion protein comprises prior to maturation either the signal sequence or the signal sequence followed by at least one membrane-spanning domain of OmpA, located N-terminal to the therapeutic molecule. The signal sequence is cleaved and absent from the mature protein. In another aspect of the embodiment, the therapeutic molecule is at the N-terminus of the OmpA fusion, rendering inconsequential to the positioning of the therapeutic molecule the number of membrane spanning domains of OmpA utilized, as long as the fusion protein is stable. In yet another aspect of the embodiment, the therapeutic molecule is situated between the N - and C-terminal domains of OmpA such that a soluble periplasmic protein containing the therapeutic molecule upon

cleavage by a periplasmic protease within the periplasm. In certain aspects of this embodiment, it is preferred that a tumor-targeted bacteria which expresses a periplasmic therapeutic molecule also coexpresses BRP to enhance release of the therapeutic molecule from the bacterial cell.

In another embodiment of the invention, the portion of the OmpA fusion protein containing the therapeutic molecule is at the extracellular bacterial surface. In one aspect of the embodiment, the fusion protein comprises an even number or odd number of membrane-spanning domains of OmpA located N-terminal to the therapeutic molecule. In another aspect of the embodiment, the therapeutic molecule is situated between two extracellular loops of OmpA for presentation to the tumor cell by the bacterial cell. In specific embodiments, the invention provides expression plasmids of therapeutic molecule fusion proteins at the bacterial extracellular surface. For example, the plasmid denoted Trc(lpp)*ompA*, comprises a *trc* promoter-driven lipopolyprotein (lpp) anchor sequence fused to a truncated *ompA* transmembrane sequence. As another example, the plasmid is denoted Trc*ompA* comprises a *trc* promoter-driven *ompA* encoding signal sequence. Such plasmids may be constructed to comprise a nucleic acid encoding one or more therapeutic molecule(s) of the invention.

Optionally, a therapeutic molecule is preceded or flanked by consensus cleavage sites for a metalloprotease or serine protease that is abundant in tumors, for release of the therapeutic molecule into the tumor environment. Whether the therapeutic molecule is preceded or flanked by protease cleavage sites depends on whether it is located terminally or internally in the fusion protein, respectively.

Similar fusion proteins may be constructed with any of the Omp-like proteins using the strategies described above in terms of OmpA. In the construction of such fusion proteins, as will be apparent to one of ordinary skill in the art, the selection of the portion of the Omp-like protein to be fused to a therapeutic molecule will depend upon the location that is desired for the expression of the therapeutic molecule (*e.g.*, periplasmic, extracellular, membrane bound, *etc.*).

In a preferred embodiment, a therapeutic molecule is fused to a ferry peptide. Ferry peptides used in fusion proteins have been shown to facilitate the delivery of a polypeptide or peptide of interest to virtually any cell within diffusion limits of its production or introduction (see., *e.g.*, Bayley, 1999, Nature Biotechnology 17:1066-1067; Fernandez *et al.*, 1998, Nature Biotechnology 16:418-420; and Derossi *et al.*, 1998, Trends Cell Biol. 8:84-87). Accordingly, engineering tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, to express fusion proteins comprising a ferry peptide and a therapeutic

molecule enhances the ability of a therapeutic molecule to be internalized by tumor cells. In a specific embodiment, tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, are engineered to express a nucleic acid molecule encoding a fusion protein comprising a ferry peptide and a therapeutic molecule. In another embodiment, tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, are engineered to express one or more nucleic acid molecules encoding one or more fusion proteins comprising a ferry peptide and a therapeutic molecule. Examples of ferry peptides include, but are not limited to, peptides derived from the HIV TAT protein, the antennapedia homeodomain (penetratin), Kaposi fibroblast growth factor (FGF) membrane-translocating sequence (MTS), herpes simplex virus VP22, polyhistadine (*e.g.*, hexahistadine; 6H), polylysine (*e.g.*, hexalysine; 6K), and polyarginine (*e.g.*, hexaarginine; 6R) (see, *e.g.*, Blanke *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93:8437-8442).

In another preferred embodiment, a fusion protein comprises a signal peptide, ferry peptide and a therapeutic molecule. In a specific mode of this embodiment, tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, are engineered to express one or more nucleic acid molecules encoding one or more fusion proteins comprising a signal sequence, a ferry peptide and a therapeutic molecule.

In another preferred embodiment, a fusion protein comprises a signal peptide, a protolytic cleavage site, a ferry peptide and a therapeutic molecule to a solid tumor by tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria,. In a specific embodiment, tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, are engineered to express one or more nucleic acid molecules encoding one or more fusion proteins comprising a signal sequence, a protolytic cleavage site, a ferry peptide and a therapeutic molecule.

By way of non-limiting example, colicin activity may be enhanced by addition of internalizing peptides derived from HIV TAT, herpes simplex virus VP22, antennapaedia, 6H, 6K, and 6R. The fusion can be either C-terminal, N-terminal, or internal. Internal fusions are especially preferred where the fusion follows the N-terminal signal sequence cleavage peptide. The fusion protein may further comprise an N-terminal signal sequence such OmpA or a C-terminal signal sequence such as hlyA.

In a preferred embodiment, a therapeutic molecule is fused to the delivery portion of a toxin. Various toxins are known to have self-delivery capacity, where one portion of the toxin acts as a delivery agent for the second portion of the toxin. For example, Ballard *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93:12531-12534 demonstrated that the anthrax protective agent (PA) which mediates the entry of lethal factor (LF) and edema factor into

the cytosolic compartment of mammalian cells, is also capable of mediating entry of protein fusions to a truncated form of LF (LFn; 255 amino acid residues). Thus, therapeutic molecules of the invention, except those that function outside the cell, can be fused to the LFn, or other toxin systems, including, but limited to, diptheria toxin A chain residues 1-193 (Blanke *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93:8437-8442), cholera toxin, verotoxin, *E. coli* heat labile toxins (LTs), *E. coli* heat stable toxins (STs), entero-hemolysins, enterotoxins, cytotoxins, EAggEC stable toxin 1 (EAST), CNFs, cytolethal distending toxin, α-hemolysins, β-hemolysins, and *SheA* hemolysins (for review see, *e.g.*, O'Brien and Holmes, 1996. Protein toxins of *Escherichia coli* and Salmonella. Cellular and Molecular Biology, Neidhardt et al. (eds), ASM Press, Washington, D.C., pp2788-2802).

Construction of fusion proteins for expression in bacteria are well-known in the art and such methods are within the scope of the invention. (See, *e.g.*, Makrides, S., 1996, Microbiol. Revs 60:512-538 which is incorporated herein by reference in its entirety).

The invention provides nucleic acid molecules encoding a therapeutic molecule. The invention also provides nucleic acid molecules encoding one or more therapeutic molecules. The invention further provides nucleic acids encoding one or more therapeutic molecules of the invention which are operably linked to an appropriate promoter. In one embodiment of the invention, the nucleic acid sequences which encode the therapeutic molecule are operatively linked to an inducible transcriptional control element. In a specific embodiment of the invention, the inducible transcriptional control element responds to conditions associated with the solid tumor cancer. By way of example, said condition can be hypoxia. Optionally, the nucleic acids encoding a therapeutic molecule may be operably linked to other elements that participate in transcription, translation, localization, stability and the like.

The nucleic acid molecule encoding a therapeutic molecule is from about 5 to about 200,000, from about 5 to about 150,000, from about 5 to about 125,000, from about 5 to about 100,000, from about 5 to about 75,000, from about 5 to about 50,000, from about 5 to about 25,000, from about 5 to about 10,000, from about 5 to about 5,000, from about 5 to about 1,000, from about 5 to about 500, or from about 5 to about 100 base pairs in length. Preferably, the nucleic acid is from about 20 base pairs to about 50,000 base pairs in length. More preferably, the nucleic acid molecule is from about 20 base pairs to about 10,000 base pairs in length. Even more preferably, the nucleic acid molecule is about 20 base pairs to about 4000 base pairs in length.

The therapeutic molecule of the invention can be a nucleotide sequences with an orientation opposite to the orientation of the coding nucleotide sequence. Such a nucleotide

sequence whose orientation is opposite to the orientation of the coding sequence is termed an antisense nucleotide sequence. Antisense nucleotides are oligonucleotides that bind in a sequence-specific manner to nucleic acids, such as mRNA or DNA. When bound to mRNA that has complementary sequences, antisense prevents translation of the mRNA (see, *e.g.*, U.S. Patent Nos. 5,168,053; 5,190,931; 5,135,917; and 5,087,617). Triplex molecules refer to single DNA strands that bind duplex DNA forming a colinear triplex molecule, thereby preventing transcription (see, *e.g.*, U.S. Patent No. 5,176,996).

The therapeutic molecule of the invention can be a ribozyme. A ribozyme is an RNA molecule that specifically cleaves RNA substrates, such as mRNA, resulting in inhibition or interference with cell growth or expression. There are at least five known classes of ribozymes involved in the cleavage and/or ligation of RNA chains. Ribozymes can be targeted to any RNA transcript and can catalytically cleave that transcript (see, *e.g.*, U.S. Patent No. 5,272,262; U.S. Patent No. 5,144,019; and U.S. Patent Nos. 5,168,053, 5,180,818, 5,116,742 and 5,093,246).

The invention further encompasses tumor-targeted bacteria that are modified to encode or deliver a fragment, derivative, analog, or variant of a therapeutic molecule, or a nucleic acid encoding the same. The fragment, derivative, analog, or variant of the therapeutic molecule is functionally active, *e.g.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type therapeutic molecule. As one example, such fragments, derivatives, analogs, or variants of a therapeutic molecule which have the desired therapeutic properties can be used to inhibit tumor growth or the spread of tumor cells (metastasis). Fragments, derivatives, analogs, or variants of a therapeutic molecule can be tested for the desired activity by procedures well-known in the art, including those described herein.

In particular, variants can be made by altering therapeutic molecule encoding sequences by substitutions, additions (*e.g.*, insertions) or deletions that provide molecules having the same or increased anti-neoplastic activity relative to the wild-type therapeutic molecule. For example, the variants of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a therapeutic molecule, including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change, *i.e.*, the altered sequence has at least one conservative substitution.

Any of the therapeutic-encoding nucleic acids that are of mammalian origin can be altered to employ bacterial codon usage by methods known in the art. Preferred codon usage is exemplified in Current Protocols in Molecular Biology, Green Publishing

Associates, Inc., and John Wiley & Sons, Inc. New York, and Zhang et al., 1991, Gene 105:61-72.

In a specific embodiment, a derivative, analog or variant of a therapeutic molecule comprises a nucleotide sequence that hybridizes to the nucleotide sequence encoding the therapeutic molecule, or fragment thereof under stringent conditions, *e.g.*, hybridization to filter-bound DNA in 6x sodium chloride/sodium citrate (SSC) at about 45 °C followed by one or more washes in 0.2xSSC/0.1% SDS at about 50-65 ° C, under highly stringent conditions, *e.g.*, hybridization to filter-bound nucleic acid in 6xSSC at about 45 °C followed by one or more washes in 0.1xSSC/0.2% SDS at about 68 °C, or under other stringent hybridization conditions which are known to those of skill in the art (see, for example, Ausubel, F.M. et al., eds. , 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York at pages 6.3.1-6.3.6 and 2.10.3).

Derivatives or analogs of a therapeutic molecule include, but are not limited to, those molecules comprising regions that are substantially homologous to the therapeutic molecule or fragment thereof (*e.g.,* in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size without any insertions or deletions or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of hybridizing to a therapeutic molecule encoding sequence, under high stringency, moderate stringency, or low stringency conditions.

To determine the percent identity of two amino acid sequences or of two nucleic acids, *e.g.* between the sequences of a therapeutic molecule and other known sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical positions/total # of positions (*e.g.*, overlapping positions) x 100). In one embodiment, the two sequences are the same length.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical

algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.*, 1990, J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997, Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Additional algorithms for sequence analysis are known in the art and include ADVANCE and ADAM as described in Torellis and Robotti, 1994, Comput. Appl. Biosci., *10*:3-5; and FASTA described in Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. *85*:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search. If ktup=2, similar regions in the two sequences being compared are found by looking at pairs of aligned residues; if ktup=1, single aligned amino acids are examined. ktup can be set to 2 or 1 for protein sequences, or from 1 to 6 for DNA sequences. The default if ktup is not specified is 2 for proteins and 6 for DNA.

Alternatively, protein sequence alignment may be carried out using the CLUSTAL W algorithm, as described by Higgins *et al.*, 1996, Methods Enzymol. 266:383-402.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

A therapeutic molecule or fragment, derivative, analog, or variant thereof can be produced by various methods known in the art. The manipulations which result in their

production can occur at the nucleic acid or protein level. For example, the nucleotide sequence encoding a therapeutic molecule can be modified by any of numerous strategies known in the art (Sambrook *et al.*, 1989, *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of a modified therapeutic molecule encoding a derivative or analog of a therapeutic molecule, care should be taken to ensure that the modified therapeutic molecule encoding sequence remains within the same translational reading frame as the native protein, uninterrupted by translational stop signals, in the therapeutic molecule encoding sequence region where the desired therapeutic molecule activity is encoded.

Additionally, a nucleic acid sequence encoding a therapeutic molecule can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or to form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. In aeferred specific embodiment, a therapeutic molecule-encoding nucleic acid sequence is mutated, for example, to produce a more potent variant. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson et al., 1978, J. Biol. Chem. 253:6551), use of TAB® linkers (Pharmacia), PCR with primers containing a mutation, *etc.* In a preferred embodiment, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues of a therapeutic molecule. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed and the activity of the protein can be determined.

In other embodiments, the therapeutic molecules of the invention are constructed to contain a protease cleavage site.

The nucleotide sequences of therapeutic molecules can be obtained or derived from any source well-known to one of skill in the art including, but not limited to, the literature and databases (*see* GenBank). A nucleic acid molecule encoding a therapeutic molecule, which is a cytotoxic or cytostatic factor or a functionally fragment, variant, analog or derivative thereof, may be isolated by standard methods, such as amplification (*e.g.*, PCR), probe hybridization of genomic or cDNA libraries, antibody screening of expression libraries, chemically synthesized or obtained from commercial or other sources.

Nucleic acid molecules and oligonucleotides for use as described herein can be synthesized by any method known to those of skill in this art (see, *e.g.*, International Publication WO 93/01286, and U.S. Patent Nos. 5,218,088; 5,175,269; and 5,109,124). Identification of oligonucleotides and ribozymes for use as antisense agents involve methods well-known in the art.

5.3.1. FACTORS PROMOTING THE RELEASE OF THERAPEUTIC MOLECULES INTO THE TUMOR ENVIRONMENT

In certain embodiments of the invention, the tumor-targeted bacteria of the methods and compositions of the invention, which express at least one therapeutic molecule express at least one auxiliary molecule which functions to permeabilize the bacteria cell membrane or enhance the release of intracellular components into the extracellular environment, *e.g.* at the tumor site, thereby enhancing the delivery of the therapeutic molecule(s). Such an auxiliary molecule which permeabilizes the bacterial cell or enhances release is designated "a release factor". In certain embodiments, the release factor also advantageously has anti-neoplastic activity.

The release factor expressed by the tumor-targeted bacteria used in the methods and compositions of the invention may be endogenous to the modified tumor-targeted bacteria or it may be exogenous (*e.g.*, encoded by a nucleic acid that is not native to the tumor-targeted bacteria). A release factor may be encoded by a nucleic acid comprising a plasmid, or by a nucleic acid which is integrated into the genome of the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria. A release factor may be encoded by the same nucleic acid or plasmid that encodes a therapeutic molecule, or by a separate nucleic acid or plasmid. In one embodiment, the release factor is expressed in a cell which also expresses a fusion protein comprising a therapeutic molecule fused to an Omp-like protein. In this

embodiment, the co-expression of the release factor allows for enhanced release of the fusion protein from the periplasmic space.

In a preferred embodiment, the release factor is one of the Bacteriocin Release Proteins, or BRPs (herein referred to in the generic as BRP). The BRP employed in the invention can originate from any source well-known in the art including, but not limited to, the cloacin DF13 plasmid, one of colicin E1-E9 plasmids, or from colicin A, N or D plasmids. In a preferred embodiment, the BRP is of cloacin DF13 (pCloDF13 BRP).

Generally, BRPs are 45-52 amino acid peptides that are initially synthesized as precursor molecules (PreBRP) with signal sequences that are not cleaved by signal endopeptidases. BRP activity is thought to be mediated, at least in part, by the detergent-resistant outer membrane phopholipase A (PldA) and is usually associated with an increase in the degradation of outer membrane phospholipid (for a general review on BRPs, see van der Wal *et al.*, 1995, FEMS Microbiology Review 17:381-399). Without limitation as to mechanism, BRP promotes the preferential release of periplasmic components, although the release of cytoplasmic components is also detected to a lesser extent. When moderatelyoverexpressed, BRP may cause the bacterial membrane to become fragile, inducing quasi-lysis and high release of cytoplasmic components. Additionally, it is thought that when BRP is expressed at superhigh levels, the protein can cause bacterial cell lysis, thus delivering cellular contents by lytic release. In this embodiment, BRP expression may be correlated with BRP activity (*e.g.*, release of bacterial contents). For example, superhigh BRP activity results in bacterial cell lysis of substantially all bacteria. Thus, as used herein, "superhigh expression" is defined as the expression level of BRP which results in bacterial cell lysis of substantially all bacteria. Moderate BRP activity, is associated with partial or enhanced release of bacterial contents as compared to a control bacteria which is not expressing BRP, without obligate lysis of the bacteria. Thus, in this embodiment, moderate overexpression of BRP is defined as the expression level at which release of cytoplasmic components is enhanced, without bacterial lysis of substantially all of the bacteria. Substantially all of the bacteria, as used herein, is more than 60% of the bacteria, preferably more than 70%, more preferably 80%, still more preferably more than 90% and most preferably 90-100% of bacteria.

In a specific embodiment of the invention, the BRP protein is a pCloDF13 BRP mutant whose lytic function has been uncoupled from its protein release function, thereby enhancing protein release without bacterial lysis (van der Wal *et al.*, 1998, App. Env. Microbiol. 64:392-398). This embodiment allows for prolonged protein release from the tumor-targeted bacteria, while reducing the need for frequent administration of the tumor-

targeted bacteria. In another specific embodiment, the BRP of the invention is a pCloDF13 BRP with a shortened C-terminus, which in addition to protein release causes cell lysis (Luirink *et al.*, 1989, J. Bacteriol. 171:2673-2679).

In a preferred embodiment of the invention, a BRP encoding nucleic acid is encoded by a colicin plasmid. In another specific embodiment of the invention, the BRP encoding nucleic acid is expressed under the control of the native BRP promoter, which is an SOS promoter that responds to stress (*e.g.*, conditions that lead to DNA damage such as UV light) in its normal host (for BRP, *Enterococcus cloacae*), yet is partially constitutive in *Salmonella*. In a preferred embodiment, the BRP encoding nucleic acid is expressed under the control of the pepT promoter, which is activated in response to the anaerobic nature of the tumor environment (*see e.g.*, Lombardo et al., 1997, J. Bacteriol. 179:1909-17).

In another embodiment, the BRP encoding nucleic acid is in a plasmid whose expression is inducible upon mitomycin C addition, or other DNA damaging agents which induce the SOS response. To determine if expression of BRP in *Salmonella* will result in the release of protein from the bacteria, the *Salmonella* BRP strains can be co-transfected with TNFα expressing plasmid, or optionally, any therapeutic protein or peptide.

Alternatively, the promoter can be an antibiotic-induced promoter, such as the *tet* promoter of the Tn10 transposon. In a preferred embodiment, the *tet* promoter is a singlemer, which singlemer responds in an all-or-nothing manner to the presence of tetracycline or analogs thereof such as doxicycline and anhydrotetracycline and provides a genetically stable on-off switch. In another embodiment, the *tet* promoter is multimerized, for example three-fold. Such a multimer responds in a graded manner to the presence of tetracycline and provides a more manipulable system for control of therapeutic molecule levels. Promoter activity would then be induced by administering to a subject who has been treated with the tumor-targeted bacteria of the invention an appropriate dose of tetracycline. Although the *tet* inducible expression system was initially described for eukaryotic systems such as *Schizosaccharomyces pombe* (Faryar and Gatz, 1992, Current Genetics 21:345-349) and mammalian cells (Lang and Feingold, 1996, Gene 168:169-171), recent studies extend its applicability to bacterial cells. For example, Stieger et al. (1999, Gene 226:243-252) have shown 80-fold induction of the firefly luciferase gene upon *tet* induction when operably linked to the *tet* promoter. An advantage of this promoter is that it is induced at very low levels of tetracycline, approximately 1/10th of the dosage required for antibiotic activity.

In another embodiment of the invention, the enhanced release system comprises overexpression of a porin protein; *see e.g.*, Sugawara, E. and Nikaido, H., 1992, J. Biol. Chem.267:2507-11.

5.4. EXPRESSION VEHICLES

The present invention provides tumor-targeted bacteria, preferably attentuated tumor targeted bacteria, encoding one or more therapeutic molecules. The invention provides tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria comprising one or more therapeutic molecules which are encoded by a plasmid or transfectable nucleic acid. In a preferred embodiment of the invention, the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, is *Salmonella*. When more than one therapeutic molecule is expressed in tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria,, such as *Salmonella*, the therapeutic molecules may be encoded by the same plasmid or nucleic acid, or by more than one plasmid or nucleic acid molecule. The invention also provides compositions and methods for use of tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, comprising one or more therapeutic molecules which are encoded by one or more nucleic acid molecules which are integrated into the bacterial genome. Integrated therapeutic molecules may be endogenous to tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, such as *Salmonella*, or may be introduced into the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria (*e.g.*, by introduction of nucleic acid molecules which encode the therapeutic molecules, such as a plasmid, transfectable nucleic acid, transposon, etc.) such that the nucleic acid molecules encoding the therapeutic molecules become integrated into the genome of the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria. In a preferred embodiment of the invention, the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, is *Salmonella*. The invention provides a nucleic acid molecule encoding a therapeutic molecule which nucleic acid is operably linked to an appropriate promoter. A promoter operably linked to a nucleic acid molecule encoding a therapeutic molecule may be homologous (*i.e.*, native) or heterologous (*i.e.*, not native to the nucleic acid molcule encoding the therapeutic molecule).

The nucleotide sequence coding for a therapeutic molecule of the invention or a functionally active analog, variant, fragment or derivative thereof, can be inserted into an appropriate expression vehicle, *e.g.*, a plasmid which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can be supplied by the therapeutic molecule and/or

its flanking regions. Alternatively, an expression vehicle is constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter using one of a variety of methods known in the art for the manipulation of DNA. See, generally, Sambrook et al., 1989, *Molecular Biology: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY; Ausubel et al., 1995, *Current Protocols in Molecular Biology*, Greene Publishing, New York, NY. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). The invention provides a nucleic acid molecule encoding a therapeutic molecule which nucleic acid is operably linked to an appropriate promoter.

The present invention also provides tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, which have been modified to encode one or more fusion proteins and optionally, one or more non-fusion-protein therapeutic molecules. The invention provides attenuated tumor-targeted bacteria comprising fusion proteins which are encoded by a plasmid or transfectable nucleic acid. When more than one therapeutic molecule is expressed in tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, such as *Salmonella*, the therapeutic molecules may be encoded by the same plasmid or nucleic acid, or by more than one plasmid or nucleic acid. The invention also provides tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, comprising fusion proteins which are encoded by a nucleic acid which is integrated into the bacterial genome. The invention also provides a nucleic acid molecule encoding an fusion protein which nucleic acid molecule is operably linked to an appropriate promoter. The nucleotide sequence encoding a fusion protein of the invention can be inserted into an appropriate expression vehicle, *e.g.*, a plasmid which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence.

In certain specific embodiments of the invention, the expression vehicle of the invention is a plasmid. Large numbers of suitable plasmids are known to those of skill in the art and are commercially available for generating the recombinant constructs of the present invention. Such commercial plasmids include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM 1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. pBR322 is considered to be a low copy number plasmid. If higher levels of expression are desired, the plasmid can be a high copy number plasmid, for example a plasmid with a pUC backbone. pUC plasmids include, but are not

limited to, pUC19 (*see e.g.*, Yanisch-Perron et al. 1985, Gene 33:103-119) and pBluescript (Stratagene).

The following plasmids are provided by way of example and may be used in conjunction with the methods of the invention: pBs, phagescript, phiX174, pbluescript SK, pBs KS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene), pCET, pTS, pGEM, pTrc99A, pKK223-3, pKK233-3, pDR540, and pRIT5 (Pharmacia). A commercial plasmid with a pBR322 "backbone" such as, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM 1 (Promega Biotec, Madison, WI, USA) may also be used in conjunction with the methods of the invention. These are combined with an appropriate promoter and the structural sequence to be expressed.

In a specific embodiment of the invention, a fusion protein for secretion into the periplasmic space of a bacteria comprising the OmpA signal sequence and a therapeutic protein is encoded by the plasmid pIN-III-*ompA*-Hind, which contains the DNA sequence encoding the ompA signal sequence upstream of a linker sequence into which the coding sequence for the therapeutic molecule can be cloned. In a preferred specific embodiment, the *lac* inducible promoter of pIN-III-*ompA*-Hind vector is replaced by a pepT or tet promoter. (See, Rentier-Delrue et al. (1988), Nuc. Acids Res. 16:8726).

The present invention also provides transposon-mediated chromosomal integration of therapeutic molecules. Any transposon plasmid known in the art may be used in the methods of the invention so long as a nucleic acid encoding a therapeutic molecule can be constructed into the transposon cassette. For example, the invention provides a transposon plasmid, comprising a transposon or minitransposon, and a multiple cloning site (MCS).

In certain embodiments of the invention, the plasmid of the invention is a transposon plasmid, *i.e.*, comprises a transposon in which the sequence encoding a therapeutic molecule of interest is inserted. Transposon plasmids contain trasposon cassettes which cassette become integrated into the bacterial genome. Accordingly, a nucleic acid encoding a therapeutic molecule is inserted into the transposon cassette. Thus, a transposon insertion integrates the cassette into the bacterial genome. The coding sequence of the therapeutic molecule can be operably linked to a promoter, or can be promoterless. In the latter case, expression of the selectable marker is driven by a promoter at the site of transposon insertion into the bacterial genome. Colonies of bacteria having a transposon insertion are screened for expression levels that meet the requirements of the invention, *e.g.* that express sufficient levels of cytokine to promote tumor cytotoxicity, stasis, or regression.

In certain embodiments, in addition to the transposon, the transposon plasmid comprises, outside the inverted repeats of the transposon, a transposase gene to catalyse the insertion of the transposon into the bacterial genome without being carried along with the transposon, so that bacteria with stable transposon insertions are generated.

Transposons to be utilized by the present invention include, but are not limited to, Tn7, Tn9, Tn10 and Tn5. In a preferred embodiment, the transposon plasmid is pNK2883 (ATCC) having an ampicillin resistance gene located outside the Tn10 insertion elements and the nucleic acids encoding one or more therapeutic molecules is inserted between the two Tn10 insertion elements (*e.g.*, within the transposon cassette). Preferably, the construct is made such that additional sequences encoding other elements is inserted between the two Tn10 insertion elements. In specific embodiments, such elements may optionally include (1) a promoterless copy a selectable marker (*e.g.*, SerC, AroA, etc) for positive selection of the bacteria containing the plasmid; (2) a BRP gene; (3) a promoter for the therapeutic molecule (such as trc) operably linked to the nucleic acid encoding the one or more therapeutic molecules; (4) a terminator for the nucleic acid encoding the one or more therapeutic molecules.

In one embodiment, after the manipulation of the plasmid as appropriate and selection of those clones having the desired construct using the ampicillin resistance properties encoded by the plasmid, the antibiotic selection is removed through plasmid loss and bacteria having a chromosomal transposon insert are chosen for administering to human subjects (*e.g.*, by plating on selective media).

In another specific embodiment, the plasmid pTS is used in accordance with the invention which comprises an altered target specificity transposase gene and a minitransposon, containing the coding sequences for a promoterless *serC* gene and an MCS. In another specific embodiment, the plasmid pTS-BRP is used in accordance with the invention which comprises an altered target specificity transposase gene and a minitransposon, containing the coding sequences for a promoterless *serC* gene, and alkylating agent-inducible bacteriocin release factor, and an MCS.

In a preferred embodiment, a transposon plasmid for selection of transposon-mediated chromosomal integrants, comprises:

a) a transposase gene, for transposon excision and integration, located outside of the transposon insertion sequence (*e.g.*, outside of the transposon cassette);

b) a wild-type coding sequence corresponding to the selection gene deleted in the bacterial strain (*e.g.*, serC) as well as a ribosomal binding site and

terminator for the wild-type gene, but lacking a promoter. This sequence is preferably located immediately following the left TN10 transposon insertion sequence;

c) optionally, between the right and left insertion sequences is a nucleic acid sequence encoding a release enhancing nucleic acid (*e.g.*, BRP); and

d) a multiple cloning site (MCS) located between the right and left insertion sequences, containing unique restriction sites within the plasmid, for the incorporation of therapeutic molecule. The MCS is preferably located immediately following the release enhancing nucleic acid (if used) and just prior to the right TN10 insertion sequence.

In another embodiment, the gene disruption resulting from random integration of therapeutic molecules onto the host chromosome, identifies the suitability of the gene location for therapeutic insertion.

In yet another embodiment, the expression vehicle is an extrachromosomal plasmid that is stable without requiring antibiotic selection, *i.e.* is self-maintained. In one non-limiting example, the self-maintained expression vehicle is a *Salmonella* virulence plasmid.

For example, in one embodiment of the invention, the plasmid selection system is maintained by providing a function which the bacteria, such as *Salmonella*, lacks and on the basis of which those *Salmonella* having the function can be selected for at the expense of those that do not. In one embodiment, the *Salmonella* of the invention is an auxotrophic mutant strain and the expression plasmid provides the mutant or absent biosynthetic enzyme function. The *Salmonella* which contain the expression plasmid can be selected for by growing the cells on growth medium which lacks the nutrient that only the desired cells, *i.e.* those with the expression plasmid, can metabolize. In a highly preferred aspect of this embodiment, the *Salmonella* of the invention has an obligatory requirement for DAP (meso-diaminopimelic acid), most preferably by deletion of the *asd* gene. DAP is a component of the peptidoglycan present in the periplasm of Gram-negative bacteria, which is required for the integrity of the bacterial outer membrane. Absence of DAP results in bacterial cell lysis resulting from the loss of outer membrane integrity. The *asd* (β-aspartate semialdehyde dehydrogenase) gene encodes an enzyme in the DAP biosynthetic pathway. Gram-negative bacteria which lack *asd* function can be grown by supplying DAP to the culture media. Plasmids, *e.g.* the expression plasmids of the invention, that carry the *asd* gene sequence operably linked to a homologous or heterologous promoter can be selected for by growing Gram-negative bacteria that lack *asd* activity in the absence of DAP (see, *e.g.*, U.S. Patent No. 5,840,483 to Curtiss, III).

Other non-antibiotic selection systems are known in the art and are within the scope of the invention. For example, a selection system utilizing a plasmid comprising a stable toxin and less stable anti-toxin may be used to select for bacteria which maintain such a plasmid.

In another embodiment, the expression vehicle is an extrachromosomal plasmid that is selectable by non-antibiotic means, for example a colicin plasmid. As used herein, a colicin plasmid minimally encodes a colicin toxin and an anti-colicin, the colicin toxin being more stable than the anti-colicin, such that any bacteria which loses the colicin plasmid is killed as a result of the perdurance of the colicin toxin. In a preferred embodiment, the colicin toxin is the large subunit of ColE3 and the anti-colicin is the small subunit of ColE3.

In other embodiments of the invention, the expression vehicle is a λ vector, more specifically a lysogenic λ vector. In a preferred embodiment, the bacterial host comprising the λ vector further comprises a temperature-sensitive λ repressor which is functional at 30°C but not 37°C. Consequently, the bacterial host can be grown and manipulated *in vivo* at 30°C without expression of the therapeutic molecule which may be toxic to the bacterial cell. Upon introduction of the bacteria into the subject, the λ repressor is inactivated by normal body temperature and expression of the therapeutic molecule.

Expression of a nucleic acid sequence encoding a therapeutic molecule may be regulated by a second nucleic acid sequence so that the therapeutic molecule is expressed in a bacteria transformed with the recombinant DNA molecule. For example, expression of a therapeutic molecule may be controlled by any promoter/enhancer element known in the art. A promoter/enhancer may be homologous (*i.e.*, native) or heterologous (*i.e.*, not native). Promoters which may be used to control the expression of a therapeutic molecule, *e.g.* a cytokine, in bacteria include, but are not limited to prokaryotic promoters such as the β-lactamase promoter (Villa-Kamaroff *et al.*, 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *lac* promoter (DeBoer *et al.*, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25; Scientific American, 1980, 242:74-94). Other promoters emcompassed by the present invention include, but are not limited to, lacI, lacZ, T3, T7, gpt, lambda P_R, lambda P_L, trc, pagC, sulA, pol II (dinA), ruv, recA, uvrA, uvrB, uvrD, umuDC, lexA, cea, caa, recN (see, *e.g.*, Schnarr *et al.*, 1991. Biochimie 73:423-431), and colE3. In a preferred embodiment, the promoter is trc (see, *e.g.*, Amann *et al.*, 1988, Gene 69:301-15).

In a particular embodiment, in which the therapeutic molecule is a colicin expressed under the control of a SOS-responsive promoter, the attenuated tumor-targeted bacterial may be treated with x-rays, ultraviolet radiation, an alkylating agent or another DNA

damaging agent such that expression of the colicin is increased. Exemplary SOS-responsive promoters include, but are not limited to, recA, sulA, umuC, dinA, ruv, uvrA, uvrB, uvrD, lexA, cea, caa, recN, etc.

In another preferred embodiment, the promoter has enhanced activity in the tumor environment; for example, a promoter that is activated by the anaerobic environment of the tumor such as the P1 promoter of the *pepT* gene. Activation of the P1 promoter is dependent on the FNR transcriptional activator (Strauch et al., 1985, J. Bacteriol. 156:743-751). In a specific embodiment, the P1 promoter is a mutant promoter that is induced at higher levels under anaerobic conditions than the native P1 promoter, such as the pepT200 promoter whose activity in response to anaerobic conditions is induced by CRP-cAMP instead of FNR (Lombardo et al., 1997, J. Bacteriol. 179:1909-1917). In another embodiment, the anaerobically-induced promoter is used, *e.g.*, the *potABCD* promoter. *potABCD* is an operon that is divergently expressed from *pepT* under anaerobic conditions. The promoter in the *pepT* gene responsible for this expression has been isolated (Lombardo et al., 1997, J. Bacteriol. 179:1909-1917) and can be used according to the methods of the present invention.

Alternatively, the promoter can be an antibiotic-induced promoter, such as the *tet* promoter of the Tn10 transposon. In a preferred embodiment, the *tet* promoter is multimerized, for example three-fold. Promoter activity would then be induced by administering to a subject who has been treated with the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, of the invention an appropriate dose of tetracycline. Although the *tet* inducible expression system was initially described for eukaryotic systems such as *Schizosaccharomyces pombe* (Faryar and Gatz, 1992, Current Genetics 21:345-349) and mammalian cells (Lang and Feingold, 1996, Gene 168:169-171), recent studies extend its applicability to bacterial cells. For example, Stieger *et al.* (1999, Gene 226:243-252) have shown 80-fold induction of the firefly luciferase gene upon tet induction when operably linked to the *tet* promoter. An advantage of this promoter is that it is induced at very low levels of tetracycline, approximately 1/10th of the dosage required for antibiotic activity.

Once a plasmid comprising a therapeutic molecule is introduced into the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, (by, *e.g.*, any method well-known to one of skill in the art, including, but not limited to, electroporation), expression of the therapeutic molecule can be assayed by any method known in the art including but not limited to biological activity, enzyme activity, Northern blot analysis, and Western blot analysis. (See Sambrook et al., 1989, *Molecular Biology: A Laboratory Manual*, Cold

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Applicant: Amador Jesús PEREZ RODRIGUEZ

Dear Sirs,

Hereby, enclosed you will find the power of attorney Signed by Mr. Amador Jesús PEREZ RODRIGUEZ as the applicant.

Kindly acknowledge receipt of this letter and inform us about the development of this application.

Yours very truly,

Belén Martínez
International Department
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Spring Harbor Press, Cold Spring Harbor, NY; Ausubel et al., 1995, *Current Protocols in Molecular Biology*, Greene Publishing, New York, NY).

5.5. METHODS AND COMPOSITIONS FOR DELIVERY

The present invention provides compositions comprising one or more immunomodulatory agents and one or more tumor-targeted bacteria, preferably an attenuated tumor-targeted bacteria. In a preferred aspect, a composition of the invention comprises one or more immunomodulatory agents and one or more tumor-targeted bacteria comprising one or more nucleotide sequences encoding one or more therapeutic molecules. Preferably, the tumor-targeted bacteria utilized in the compositions of the invention are attenuated.

The present invention provides pharmaceutical compositions comprising pharmaceutically acceptable carriers, one or more immunomodulatory agents, and one tumor-targeted bacteria. The present invention also provides pharmaceutical compositions comprising pharmaceutically acceptable carriers, one or more immunomodulatory agents, and one or more tumor-targeted bacteria comprising one or more nucleic acid molecule encoding one or more therapeutic molecules. The present invention also provides pharmaceutical compositions comprising pharmaceutically acceptable carriers, one or more immunomodulatory agents, and one or more tumor-targeted bacteria comprising one or more nucleic acid molecules encoding one or more therapeutic molecules operably linked to one or more appropriate promoters.

The present invention provides pharmaceutical compositions comprising pharmaceutically acceptable carriers, one or more immunomodulatory agents, and one or more tumor-targeted bacteria comprising one or more nucleotide sequences encoding one or more fusion proteins and optionally, one or more non-fusion-protein therapeutic molecules. The present invention also provides pharmaceutical compositions comprising pharmaceutically acceptable carriers, one or more immunomodulatory agents, and tumor-targeted bacteria comprising one or more nucleic acid molecules encoding one or more fusion proteins of the invention operably linked to one or more appropriate promoters. The present invention also provides pharmaceutical compositions comprising pharmaceutically acceptable carriers, one or more immunomodulatory agents, and one or more tumor-targeted bacteria comprising one or more nucleic acid molecules encoding one or more fusion proteins of the invention, and optionally one or more non-fusion-protein therapeutic molecules operably linked to one or more appropriate promoters.

The pharmaceutical compositions of the invention comprise therapeutically effective amounts of tumor-targeted bacteria and therapeutically effective amounts of one or more immunomodulatory agents. The pharmaceutical compositions of the invention further comprise therapeutically effective amounts of one or more immunomodulatory agents and effective amounts of tumor-targeted bacteria to deliver a therapeutically effective amount of one or more therapeutic molecules locally to the site of solid tumor cancers. Preferably, the tumor-targeted bacteria used in the pharmaceutical compositions are attenuated. In a preferred embodiment, the tumor-targeted bacteria used in the pharmaceutical compositions are attenuated tumor-targeted *Salmonella* mutants. In another preferred embodiment, the pharmaceutical compositions of the invention are free of any living biological material except for the tumor-targeted bacteria and formulated for use in the treatment of a solid tumor cancer.

In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, olive oil, and the like. Saline is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the therapeutic tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, in purified form, and therapeutically effective amounts of one or more immunomodulatory

agents, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a suspending agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the components are supplied either separately or mixed together in unit dosage form, for example, in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of a sterile buffer for injection can be provided so that the components may be mixed prior to administration.

For administration by inhalation, the pharmaceutical compositions of the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The amount of the pharmaceutical composition of the invention which will be effective in the treatment of a solid tumor cancer will depend on the nature of the cancer and the overall state of the subject, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the cancer, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for the tumor-targeted bacteria are generally from about 1.0 c.f.u./kg to about 1×10^{10} c.f.u./kg; optionally from about 1.0 c.f.u./kg to about 1×10^{8} c.f.u./kg; optionally from about 1×10^{2} c.f.u./kg to about 1×10^{8} c.f.u./kg; optionally from about 1×10^{4} c.f.u./kg to about 1×10^{8} c.f.u./kg; and optionally from about 1×10^{4} c.f.u./kg to about 1×10^{10} c.f.u./kg. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

The dosage of the immunomodulatory agent used in accordance with the methods will depend on several aspects including, but not limited to, the kind of the immunomodulatory agent used, the general state of the patient, the indicated tumor, and the tumor-targeted bacteria. However, suitable dosage ranges for the immunomodulatory agents are generally from about 1×10^{-4} to about $1 \times 10^{+3}$ mpk (a.k.a., mg per kg of a subject's body weight). In particular, suitable dosage ranges for immunomodulatory agents that are antibodies, proteins, polypeptides, peptides and fusion proteins are typically 0.0001 mg/kg to 100 mg/kg of the subject's body weight. Preferably, the dosage of an immunomodulatory agent that is an antibody, protein, polypeptide, peptide or fusion protein that is administered to a subject with a solid tumor cancer is between 0.0001 mg/kg and 20 mg/kg, 0.0001 mg/kg and 10 mg/kg, 0.0001 mg/kg and 5 mg/kg, 0.0001 and 2 mg/kg, 0.0001 and 1 mg/kg, 0.0001 mg/kg and 0.75 mg/kg, 0.0001 mg/kg and 0.5 mg/kg, 0.0001 mg/kg to 0.25 mg/kg, 0.0001 to 0.15 mg/kg, 0.0001 to 0.10 mg/kg, 0.001 to 0.5 mg/kg, 0.01 to 0.25 mg/kg or 0.01 to 0.10 mg/kg of the subject's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention or fragments thereof may be reduced by enhancing uptake and tissue penetration of the antibodies by modifications such as, for example, lipidation.

In a specific embodiment, the dosage of an immunomodulatory agent that is an antibody, protein, polypeptide, peptide or fusion protein administered to a subject with a solid tumor cancer is 500 μg/kg or less, preferably 250 μg/kg or less, 200 μg/kg or less, 150 μg/kg or less, 125 μg/kg or less, 100 μg/kg or less, 95 μg/kg or less, 90 μg/kg or less, 85 μg/kg or less, 80 μg/kg or less, 75 μg/kg or less, 70 μg/kg or less, 65 μg/kg or less, 60 μg/kg or less, 55 μg/kg or less, 50 μg/kg or less, 45 μg/kg or less, 40 μg/kg or less, 35 μg/kg or less, 30 μg/kg or less, 25 μg/kg or less, 20 μg/kg or less, 15 μg/kg or less, 10 μg/kg or less, 5 μg/kg or less, 2.5 μg/kg or less, 2 μg/kg or less, 1.5 μg/kg or less, 1 μg/kg or less, 0.5 μg/kg or less, or 0.5 μg/kg or less of a patient's body weight. In another embodiment, the dosage of an immunomodulatory agent that is an antibody, protein, polypeptide, peptide or fusion protein administered to a subject with a solid tumor cancer is a unit dose of 0.1 mg to 20 mg, 0.1 mg to 15 mg, 0.1 mg to 12 mg, 0.1 mg to 10 mg, 0.1 mg to 8 mg, 0.1 mg to 7 mg, 0.1 mg to 5 mg, 0.1 to 2.5 mg, 0.25 mg to 20 mg, 0.25 to 15 mg, 0.25 to 12 mg, 0.25 to 10 mg, 0.25 to 8 mg, 0.25 mg to 7mg, 0.25 mg to 5 mg, 0.5 mg to 2.5 mg, 1 mg to 20 mg, 1 mg to 15 mg, 1 mg to 12 mg, 1 mg to 10 mg, 1 mg to 8 mg, 1 mg

to 7 mg, 1 mg to 5 mg, or 1 mg to 2.5 mg. In yet another embodiment, the dosage of an immunomodulatory agent that is an antibody, protein, polypeptide, peptide or fusion protein administered to a subject with a solid tumor cancer is a unit dose of 0.1 mg, 0.5 mg, 1 mg, 2 mg, 2.5 mg, 5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg or 40 mg.

Suitable dosage ranges of an immunomodulatory agent that is a chemotherapeutic agent are typically 0.01 mg/kg to 500 mg/kg of the subject's body weight. Preferably, the dosage of an immunomodulatory agent that is a chemotherapeutic agent that is administered to a subject with a solid tumor cancer is between 0.01 mg/kg and 100 mg/kg, 0.1 mg/kg and 75 mg/kg, 0.1 mg/kg and 50 mg/kg, 0.1 and 25 mg/kg, 0.1 and 10 mg/kg, 0.1 mg/kg and 5 mg/kg, 0.1 mg/kg and 2.5 mg/kg, or 0.1 mg/kg to 2 mg/kg of the subject's body weight. In a specific embodiment, the dosage of methotrexate administered to a subject with a solid tumor cancer in accordance with the methods the invention ranges from 0.01 mg/kg to 75 mg/kg, preferably 0.1 mg/kg to 50 mg/kg, 0.1 mg/kg to 25 mg/kg, 0.1 mg/kg to 10 mg/kg, 0.1 mg/kg to 5 mg/kg, 0.1 mg/kg to 2.5 mg/kg, or 0.5 mg/kg to 2 mg/kg. In a particular embodiment, the dosage of methotrexate administered to a subject with a solid tumor cancer in accordance with the methods the invention is 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 8 mg/kg or 10 mg/kg of the subject's body weight. In another embodiment, the dosage of cyclosporin A administered to a subject with a solid tumor cancer in accordance with the methods the invention ranges from 0.01 mg/kg to 75 mg/kg, preferably 0.1 mg/kg to 50 mg/kg, 0.1 mg/kg to 25 mg/kg, 0.1 mg/kg to 10 mg/kg, 0.1 mg/kg to 5 mg/kg, 0.1 mg/kg to 2.5 mg/kg of the subject's body weight. In a particular embodiment, the dosage of cyclosporin A administered to a subject with a solid tumor cancer in accordance with the methods the invention is 0.5 mg/kg, 1 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg or 10 mg/kg of the subject's body weight. In certain embodiments, the dosage of the immunomodulatory agent administered to a subject with a solid tumor cancer in accordance with the methods of the invention is analogous to the dosage of immunomodulatory agent administered to a subject with an inflammatory disorder (*e.g.*, rheumatoid arthritis and psoriasis) or a subject rejecting an organ transplant.

The frequency of administration of the dosage of a therapeutically effective amount an immunomodulatory agent will vary depending upon the route of administration, the nature of the cancer, the condition of the subject, in particular, the state of the subject's immune system, and the nature of the tumor-targeted bacteria being administered in combination with the immunomodulatory agent. In a specific embodiment, a dosage of a therapeutically effective amount of an immunomodulatory agent is given for a short period

of time proceeding the administration of a dosage of a therapeutically effective amount of a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria) to a subject with a solid tumor cancer. In another embodiment, a dosage of a therapeutically effective amount of an immunomodulatory agent is given for a short period of time proceeding and following the administration of a dosage of a therapeutically effective amount of a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria) to a subject with a solid tumor cancer. In yet another embodiment, a dosage of a therapeutically effective amount of an immunomodulatory agent is given for a short period of time proceeding the administration of a dosage of a therapeutically effective amount of a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria), during the administration of a dosage of a therapeutically effective amount of a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria) and a short period of time following the administration of a dosage of a therapeutically effective amount of a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria) to a subject with a solid tumor cancer. The term "short period of time" in this context refers to less than 5 minutes, at least 5 minutes, at least 10 minutes, at least 15 minutes, at least 30 minutes, at least 45 minutes, at least 1 hour, at least 2 hours, at least 4 hours, at least 6 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least 36 hours, at least 48 hours, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 8 days, at least 9 days, at least 10 days, or at least 14 days.

In one embodiment, a dosage of a therapeutically effective amount of an immunomodulatory agent and a dosage of a therapeutically effective amount of a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria) are administered to a subject with a solid tumor cancer on the same day. In a specific embodiment, a dosage of a therapeutically effective amount methotrexate and a dosage of a therapeutically effective amount of a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria) are administered to a subject with a solid tumor cancer on the same day. In a particular embodiment, a dosage of 0.5 mg/kg of a subject's body weight of methotrexate and a dosage of $3x10^8$ c.f.u./m2 of the surface area of a subject of an attenuated tumor-targeted bacteria (preferably, VNP20009) are administered to a subject with a solid tumor cancer.

In another embodiment, a dosage of a therapeutically effective amount of an immunomodulatory agent is administered once a week for 2, 3, 4, 5, or 6 consecutive weeks to a subject with a solid tumor cancer and a dosage of a therapeutically effective amount of a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria) is administered 1, 2, 3, 4, 5 or 6 days after the first, second, third, fourth, fifth or sixth dose of

the therapeutically effective amount of the immunomodulatory agent to a subject with a solid tumor cancer. In accordance with this embodiment, the dosage of the therapeutically effective amount of the immunomodulatory agent administered to the subject may be increased or decreased as treatment progresses. In a preferred embodiment, a unit dosage of 500 μg of an anti-CD8 antibody (preferably, an anti-CD8 monoclonal antibody) is administered once a week for 4 consecutive weeks and a dosage of $3x10^8$ c.f.u./m2 of the surface area of a subject of an attenuated tumor-targeted bacteria (preferably, VNP20009) is administered 2 days after the first, second, third or fourth dose to a subject with a solid tumor cancer.

In another embodiment, a dosage of a therapeutically effective amount of a first immunomodulatory agent is administered on 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive days, a dosage of a therapeutically effective amount of a second, different immunomodulatory agent is administered on days 2, 4, and 6 or days 2, 4, 6 and 10 of the administration of the first immunomodulatory agent, and a dosage of a therapeutically effective amount of a tumor-targeted bacteria (preferably, an attenuated tumor-targeted bacteria) is administered on day 2, 3, 4, 5, 6, 7, 8, 9 or 10 of the administration of the first immunomodulatory agent to a subject with a solid tumor cancer. In a particular embodiment, a dosage of 5 mg/kg of a subject's body weight of cyclosporin A is administered (preferably, orally) on 4 consecutive days, a dosage of 2 mg/kg of a subject's body weight of methrotexate is administered on days 2 and 4 of the administration of cyclosporin A and 2 days after the last dosage of cyclosporin A, and a dosage of $3x10^8$ c.f.u./m2 of the surface area of a subject of an attenuated tumor-targeted bacteria (preferably, VNP20009) is administered on day 2 of the cyclosporin A treatment to a subject with a solid tumor cancer.

Various delivery systems are known and can be used to administer a pharmaceutical composition of the present invention. Delivery systems include, but are not limited to, encapsulation in liposomes, microparticles, microcapsules, receptor-mediated endocytosis (*see*, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432). Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intrathecal, intranasal, epidural, and oral routes. Methods of introduction may also be intratumoral (*e.g.*, by direct administration into the area of the tumor). Pharmaceuticals of the invention may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents such as chemotherapeutic agents. Administration can be systemic or local.

In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer one or more of the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

The present invention provides methods of treating a solid tumor cancer in a subject, said methods comprising administering to said subject one or more immunomodulatory agents and one or more tumor-targeted bacteria. The present invention also provides methods of treating a solid tumor cancer in a subject, said methods comprising administering to said subject one or more immunomodulatory agents and one or more tumor-targeted bacteria comprising one or more nucleic acid molecules encoding one or more therapeutic molecules. In a specific embodiment, one or more immunomodulatory agents are administered prior to (*e.g.*, 2 hours, 6 hours, 12 hours, 1 day, 4 days, 6 days, 12 days or 14 days before) the administration of tumor-targeted bacteria. In another specific embodiment, one or more immunomodulatory agents are administered subsequent to (*e.g.*, 2 hours, 6 hours, 12 hours, 1 day, 4 days, 6 days, 12 days, or 14 days after) the administration of tumor-targeted bacteria. In another specific embodiment, one or more immunomodulatory agents are administered concomitantly with tumor-targeted bacteria. In accordance with this embodiment, the immunomodulatory agents and tumor-targeted bacteria can be administered separately or as an admixture. Preferably, the tumor-targeted bacteria used in accordance with the methods of the invention are attenuated. In a preferred embodiment, the tumor-targeted bacteria used in accordance with the methods of the invention are attenuated tumor-targeted *Salmonella*.

The present invention provides for the use of combinations of different immunomodulatory agents with different tumor-targeted bacteria for the treatment of a solid tumor cancer in a subject. Combinations of immunomodulatory agents and tumor-targeted bacteria can be administered concomitantly or at different times. If

immunomodulatory agents and/or tumor-targeted bacteria are administered repeatedly to a subject with a solid tumor cancer, the immunomodulatory agent and the tumor-targeted bacteria can be administered concomitantly or at different times. The procedure can be started either with the administration of the tumor-targeted bacteria or with the administration of one or more immunomodulatory agent.

Examples of the types of cancers which can be treated utilizing the methods of the invention include, but are not limited to, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma. Additional examples of solid tumors which can be treated utilizing the methods of the invention include, but are not limited to, germ line tumors, tumors of the central nervous system, uterine cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, stomach cancer, liver cancer, colon cancer, renal cancer, bladder cancer, and mesothelioma.

Immunomodulatory agents and tumor-targeted bacteria can be administered to a subject with a solid tumor cancer in accordance with the methods of the invention by any drug delivery system well-known to the skilled artisan. Said drug delivery systems include, but are not limited to, intratumoral injection, intravenous injection, subcutaneous injection, intramuscular injection, intraperitoneal injection, oral administration, sustained release systems and controlled release systems. In a one embodiment of the invention, the tumor-targeted bacteria, preferably the attenuated tumor-targeted bacteria, are administered to a subject with a solid tumor cancer utilizing a controlled release system or sustained delivery system. More specifically, an implant comprising said tumor-targeted bacteria is implanted close to the site of the solid tumor cancer. The implant may be composed of a porous, non-porous, or gelatinous material, including a membrane, such as a sialastic membrane, or a fiber. Preferably, the implant is composed of a porous material whose pore size

corresponds to the diameter of said bacteria. Prior to implanting the implant, the pores of the implant are filled with said tumor-targeted bacteria. Once implanted, the tumor-targeted bacteria diffuse out of the implant. The release of bacteria from the implant to the subject can take hours, days, weeks, or months. In this embodiment of the invention, one or more immunomodulatory agents are administered either together with the tumor-targeted bacteria or separately. If the immunomodulatory agents are administered separately, the immunomodulatory agents can be administered via any delivery system well-known to the skilled artisan, such as, but not limited to, intratumoral injection, intravenous injection, subcutaneous injection, intramuscular injection, intraperitoneal injection, oral administration, a controlled release system or a sustained delivery system.

In a specific embodiment of the invention, one or more immunomodulatory agents are administered to a subject with a solid tumor cancer via a sustained release system or a controlled-release drug delivery system. See, *e.g.*, Langer, 1990, Science 249:1527-1533; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., 1980, Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321:574; Medical Applications of Controlled Release, Langer and Wise (eds)., CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1983); Ranger and Peppas, 1983, Macromol. Sci. Rev. Macromol. Chem. 23:61; Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351; and Howard et al., 1989, J. Neurosurg. 71:105 for discussion of controlled release systems. In another embodiment, one or more immunomodulatory agents are administered to a subject with a solid tumor cancer by a vesicle, in particular a liposome (*see* Langer, 1990, Science 249:1527-1533; Treat *et al.*, 1989, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365; Lopez-Berestein, ibid., pp. 317-327; see generally, *ibid.*).

In a specific embodiment, nucleotide sequences encoding immunomodulatory agents, such as antisense RNA, ribozymes, proteins, polypeptides and peptides are administered via a gene therapy approach to modify the immune response in a subject with a solid tumor cancer. In a specific embodiment of the invention, nucleotide sequences encoding antibodies specific to CD3, CD4, or CD8 are administered to a subject with a solid tumor cancer via gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleotide sequence. In this embodiment of the invention, the nucleotide sequences encode proteins or RNA molecules that mediate an immunomodulatory effect.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the method of gene therapy, *see*, Goldspiel *et al.*, 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 1, l(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); and Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

In a specific embodiment, nucleic acid molecules are used in which the nucleotide sequence encoding the immunomodulatory agents is flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the nucleotide sequence of the invention (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438).

In certain embodiments, the nucleic acid sequences encoding the immunomodulatory agents are directly administered *in vivo*, where they are expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, for example by constructing them as part of an appropriate nucleic acid expression vector and administering the nucleic acid vector so that the nucleic acid sequences become intracellular. Gene therapy vectors can be administered by infection using defective or attenuated retrovirals or other viral vectors (*see*, *e.g.*, U.S. Patent No. 4,980,286); direct injection of naked DNA; use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont); coating with lipids or cell-surface receptors or transfecting agents; encapsulation in liposomes, microparticles, or microcapsules; administration in linkage to a peptide which is known to enter the nucleus; administration in linkage to a ligand subject to receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) (which can be used to target cell types specifically expressing the receptors); *etc.* In certain other embodiments, the nucleotide sequence encoding the immunomodulatory agents can be introduced into cells *ex vivo* and subsequently said cells are administered to a subject with a solid tumor cancer. The nucleotide sequence encoding the immunomodulatory agents can be introduced into said cells by any method known to the skilled artisan, including, but not limited to, electroporation and calcium phosphate transfection.

In a specific embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation.

In certain embodiments, the nucleic acids encoding the immunomodulatory agents can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (*see, e.g.*, International Publications Nos. WO 92/06180; WO 92/22635; W092/20316; W093/14188, and WO 93/20221). Alternatively, the nucleic acid encoding the immunomodulatory agents can be introduced intracellularly and incorporated within host cell DNA for expression by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438).

In a specific embodiment, viral vectors that contain the nucleotide sequence encoding the immunomodulatory agents are used. For example, a retroviral vector can be used (see Miller *et al.*, 1993, Meth. Enzymol. 217:581-599). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleotide sequences encoding an immunomodulatory agent to be used in gene therapy are cloned into one or more vectors, thereby facilitating delivery of the gene into a subject with a solid tumor cancer. More detail about retroviral vectors can be found in Boesen *et al.*, 1994, Biotherapy 6:29 1-302, which describes the use of a retroviral vector to deliver the mdr 1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes *et al.*, 1994, J. Clin. Invest. 93:644-651; Klein *et al.*, 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114. Other examples of viral vectors which can be used in gene therapy include, but are not limited to, adeno-associated virus vectors (see, *e.g.*, U.S. Patent No. 5,436,146) and adenovirus vectors.

In certain embodiments of the invention, one or more attenuated bacteria, preferably, attenuated tumor-targeted bacteria, comprising one or more nucleic acid molecules encoding one or more immunomodulatory agents (*i.e.*, one or more proteins, polypeptides, or peptides with immunomodulatory activity) are used to administer the immunomodulatory agents to a subject with a solid tumor cancer. In accordance with these embodiments, the attenuated bacteria may be attenuated version of any species of bacteria. Preferably, the attenuated bacteria are facultative anaerobes or facultative aerobes and more preferably, the attenuated bacteria are attenuated tumor-targeted bacteria that are facultative anaerobes or facultative aerobes. The attenuated tumor-targeted bacteria comprising one or

more nucleic acid molecules encoding one or more immunomodulatory agents may also comprise one or more nucleic acid molecules encoding one or more therapeutic molecules which are not immunomodulatory agents. The attenuated tumor-targeted bacteria comprising one or more nucleic acid molecules encoding one or more immunomodulatory agents may be a different strain or species than the tumor-targeted bacteria comprising one or more nucleic acid molecules encoding one or more therapeutic molecules which are not immunomodulatory agents. In certain embodiment of the invention, attenuated bacteria, preferably attenuated tumor-targeted bacteria, comprising one or more nucleic acid molecules encoding one or more immunomodulatory agents are not utilized to administer immunomodulatory agents to a subject with a solid tumor cancer.

In certain embodiments of the invention, the nucleotide sequence encoding an immunomodulatory agent is operatively linked to one or more regulatory regions, *i.e.*, another nucleotide sequence that initiates and regulates the expression of the immunomodulatory agent(s). The following animal regulatory regions, which exhibit tissue specificity and have been utilized in transgenic animals, can be used for expression in a particular tissue type: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in the liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in the liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

The present invention also provides methods for treating a solid tumor comprising administering to a subject in need thereof, one or more tumor-targeted bacteria which optionally comprise nucleotide sequences encoding one or more therapeutic molecules, one or more immunomodulatory agents, and at least one other known cancer therapy. Examples of known cancer therapies include, but are not limited to, anti-angiogenic agents (*e.g.*, endostatin, angiostatin, apomigren, anti-angiogenic antithrombin III, the 29 kDa N-terminal and a 40 kDa C-terminal proteolytic fragments of fibronectin, a uPA receptor antagonist, the 16 kDa proteolytic fragment of prolactin, the 7.8 kDa proteolytic fragment of platelet factor-4, the anti-angiogenic 24 amino acid fragment of platelet factor-4, the anti-angiogenic factor designated 13.40, the anti-angiogenic 22 amino acid peptide fragment of thrombospondin I, the anti-angiogenic 20 amino acid peptide fragment of SPARC, RGD and NGR containing peptides, the small anti-angiogenic peptides of laminin, fibronectin, procollagen and EGF, integrin $\alpha_v\beta_3$ antagonists (*e.g.*, anti-integrin $\alpha_v\beta_3$ antibodies), acid fibroblast growth factor (aFGF) antagonists, basic fibroblast growth factor (bFGF) antagonists, vascular endothelial growth factor (VEGF) antagonists, and VEGF receptor (VEGFR) antagonists (*e.g.*, anti-VEGFR antibodies)), bisphosphonates, (*e.g.*, pamidronate, sodium clondronate, aredia, bonefos, zoledronic acid, zometa, fosamax, alendronate, etidronate, pimidronate, ibandronate, cimadronate, risedronate or tiludronate), HMG CoA reductase inhibitors (*e.g.*, lescol, lipitor, lovastatin, simvastatin, atorvastatin, pravastatin, fluvastatin, cerivastatin or rosuvastatin), anti-inflammatory agents non-steroidal anti-inflammatory drugs (NSAIDs; *e.g.*, aspirin, ibuprofen, celecoxib (CELEBREX™), diclofenac (VOLTAREN™), etodolac (LODINE™), fenoprofen (NALFON™), indomethacin (INDOCIN™), ketoralac (TORADOL™), oxaprozin (DAYPRO™), nabumentone (RELAFEN™), sulindac (CLINORIL™), tolmentin (TOLECTIN™), rofecoxib (VIOXX™), naproxen (ALEVE™, NAPROSYN™), ketoprofen (ACTRON™) and nabumetone (RELAFEN™)), steroidal anti-inflammatory drugs (*e.g.*, glucocorticoids, dexamethasone (DECADRON™), cortisone, hydrocortisone, prednisone (DELTASONE™), prednisolone, triamcinolone, azulfidine, and eicosanoids such as prostaglandins, thromboxanes, and leukotrienes), beta-agonists, anticholingeric agents, and methyl xanthines), anti-cancer antibodies (*e.g.*, OvaRex (AltaRex Corporation, MA) which is a murine antibody for the treatment of ovarian cancer; Panorex (Glaxo Wellcome, NC) which is a murine IgG2a antibody for the treatment of colorectal cancer; BEC2 (ImClone Systems Inc., NY) which is murine IgG antibody for the treatment of lung cancer; IMC-C225 (Imclone Systems Inc., NY) which is a chimeric IgG antibody for the treatment of head and neck cancer; Campath I/H (Leukosite, MA) which is a humanized

IgG1 antibody for the treatment of chronic lymphocytic leukemia (CLL); Smart MI95 (Protein Design Labs, Inc., CA) which is a humanized IgG antibody for the treatment of acute myeloid leukemia (AML); LymphoCide (Immunomedics, Inc., NJ) which is a humanized IgG antibody for the treatment of non-Hodgkin's lymphoma; Smart I D10 (Protein Design Labs, Inc., CA) which is a humanized antibody for the treatment of non-Hodgkin's lymphoma; Oncolym (Techniclone, Inc., CA) which is a murine antibody for the treatment of non-Hodgkin's lymphoma; and the anti-CD20 monoclonal antibody sold by Beckman Coulter, Inc., CA)). In a specific embodiment, a subject with a solid tumor cancer is administered one or more tumor-targeted bacteria, one or more immunomodulatory agents other than a chemotherapeutic agent, and at least one chemotherapeutic agent. Examples of chemotherapeutic agents include, but are not limited to, methotrexate, cyclosporin A, leflunomide, cisplatin, ifosfamide, taxanes such as taxol and paclitaxol, topoisomerase I inhibitors (*e.g.*, CPT-11, topotecan, 9-AC, and GG-211), gemcitabine, vinorelbine, oxaliplatin, 5-fluorouracil (5-FU), leucovorin, vinorelbine, temodal, cytochalasin B, gramicidin D, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin homologs, and cytoxan.

In another embodiment, the tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, and immunomodulatory agents are used in conjunction with anti-cancer antibodies, which include, but are not limited to, polyclonal, monoclonal, bispecific, human, humanized or chimeric antibodies, single chain antibodies, sFvs, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above which immunospecifically bind to cancer cell antigens. Examples of such antibodies include, but are not limited to, Herceptin® (Trastuzumab; Genetech, CA) which is a humanized anti-HER2 monoclonal antibody for the treatment of patients with metastatic breast cancer, and the monoclonal antibody 7E11 which immunospecifically binds to prostate-specific membrane antigen (PSMA; Lin et al., 1997, Cancer Res. 57:3629). The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which immunospecifically binds a cancer cell antigen. The immunoglobulin molecules of the invention can be of any type (*e.g.*, IgG, IgE, IgM, IgD and IgA), class, or subclass of immunoglobulin molecule.

The present invention includes the sequential or concomitant administration of the pharmaceuticals of the invention and an anti-cancer agent such as a chemotherapeutic

agent. In a specific embodiment, the pharmaceutical compositions of the invention are administered prior to (*e.g.*, 2 hours, 6 hours, 12 hours, 1 day, 4 days, 6 days, 12 days, 14 days, 1 month or several months before) the administration of the anti-cancer agent. In another specific embodiment, the pharmaceutical composition of the invention is administered subsequent to (*e.g.*, 2 hours, 6 hours, 12 hours, 1 day, 4 days, 6 days, 12 days, 14 days, 1 month or several months after) the administration of an anti-cancer agent. In a specific embodiment, the pharmaceutical compositions of the invention are administered concomitantly with an anti-cancer agent. The invention encompasses combinations of anti-cancer agents and pharmaceutical compositions of the invention that have additive or synergistic effects.

The invention also encompasses combinations of anti-cancer agents, one or more immunomodulatory agents and tumor-targeted bacteria, preferably attenuated tumor-targeted bacteria, engineered to express one or more nucleic acid molecules encoding one or more therapeutic molecules that have different sites of action. Such a combination provides an improved therapy based on the dual action of these therapeutics whether the combination is synergistic or additive. Thus, the novel combinational therapy of the present invention yields improved efficacy over either agent used as a single-agent therapy.

In one embodiment, an subject with a solid tumor cancer is administered a pharmaceutical composition of the invention and treated with radiation therapy (e.g., gamma radiation or x-ray radiation). In a specific embodiment, the invention provides a method to treat or prevent cancer that has shown to be refractory to radiation therapy. The pharmaceutical composition may be administered concurrently with radiation therapy. Alternatively, radiation therapy may be administered subsequent to administration of a pharmaceutical composition of the invention, preferably at least an hour, five hours, 12 hours, a day, a week, a month, more preferably several months (*e.g.*, up to three months), subsequent to administration of a pharmaceutical composition.

The radiation therapy administered prior to, concurrently with, or subsequent to the administration of the pharmaceutical composition of the invention can be administered by any method known in the art. Any radiation therapy protocol can be used depending upon the type of cancer to be treated. For example, but not by way of limitation, x-ray radiation can be administered; in particular, high-energy megavoltage (radiation of greater that 1 MeV energy) can be used for deep tumors, and electron beam and orthovoltage x-ray radiation can be used for skin cancers. Gamma ray emitting radioisotopes, such as radioactive isotopes of radium, cobalt and other elements may also be administered to expose tissues to radiation.

Additionally, the invention provides methods of treatment of cancer with a pharmaceutical composition of the invention as an alternative to radiation therapy where the radiation therapy has proven or may prove too toxic, *i.e.*, results in unacceptable or unbearable side effects, for the subject being treated.

5.6. METHODS OF DETERMINING THE THERAPEUTIC UTILITY

Several aspects of the pharmaceutical composition of the invention are preferably tested *in vitro*, in a cell culture system, and in an animal model organism, such as a rodent animal model system, for the desired therapeutic activity prior to use in humans. For example, assays which can be used to determine whether administration of a specific pharmaceutical composition is indicated, include cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise contacted with a pharmaceutical composition, and the effect of such composition upon the tissue sample is observed. The tissue sample can be obtained by biopsy from the patient. This test allows the identification of the therapeutically most effective tumor-targeted bacteria and the therapeutically most effective therapeutic molecule(s) for each individual patient. In various specific embodiments, *in vitro* assays can be carried out with representative cells of cell types involved in a solid tumor cancer, to determine if a pharmaceutical composition of the invention has a desired effect upon such cell types.

Combinations of immunomodulatory agents and different tumor-targeted bacteria can be tested in suitable animal model systems prior to use in humans. Such animal model systems include, but are not limited to, rats, mice, chicken, cows, monkeys, pigs, dogs, rabbits, etc. Any animal system well-known in the art may be used. In a specific embodiment of the invention, combinations of immunomodulatory agents and one or more tumor-targeted bacteria are tested in a mouse model system. Such a mouse model can be genetically engineered to develop tumors or the mice can be injected with tumorigenic cells (*e.g.*, murine melanoma B16F10, human breast cancer MDA-MB-231, and human colon adenocarcinoma DLD1). Such model systems are widely used and well-known to the skilled artisan. Once the animal model is inoculated with the tumorigenic cells and/or starts to develop tumors which can be after a period of several days, weeks or months, one or more immunomodulatory agents are administered in combination with one or more tumor-targeted bacteria. Immunomodulatory agents can be administered prior to, concomitantly with, or subsequent to the administration of tumor-targeted bacteria. Immunomodulatory agents and/or tumor targeted bacteria can be administered repeatedly. Several aspects of the procedure may vary. Said aspects include the temporal regime of

administering the immunomodulatory agents, the temporal regime of administering the tumor-targeted bacteria, the kind of immunomodulatory agent, the kind of tumor-targeted bacteria, whether immunomodulatory agents and/or tumor targeted bacteria are administered repeatedly, and whether immunomodulatory agents and tumor targeted bacteria are administered seperately or as an admixture. The effectiveness of the procedure can be determined by measuring the c.f.u. of tumor targeted bacteria in the tumor over a timecourse of several days, weeks, or months. The effectiveness of the procedure can be determined by measuring the volume of the tumor, survival rates, LD_{50} and overall biodistribution of the therapeutic molecule and spread of the tumor in the animal (metastasis) over a time course of several days, weeks, or months.

Different combinations of immunomodulatory agents with different tumor-targeted bacteria can be tested for their ability to reduce tumor formation in animals suffering from cancer. Pharmaceutical compositions of the invention can also be tested for their ability to alleviate of one or more symptoms associated with a solid tumor cancer. Further, pharmaceutical compositions of the invention can be tested for their ability to increase the survival period of patients suffering from a solid tumor cancer. Techniques well-known to those of skill in the art can be used to analyze the function of the pharmaceutical compositions of the invention in animals. For example, the pharmaceutical compositions of the invention can be tested for their ability to enhance killing of tumor cells, in, *e.g.*, an *in vitro* clonogenic assay, *i.e.*, a bacteria cytotoxicity/bystander effect assay.

Another test employs an immunocompromised or immunodeficient mouse model (*e.g.* SCID or nude mice). Such mouse models are widely used, well-known to the skilled artisan, and readily available. Tumor tissue is injected into the immunocompromised or immunodeficient mouse model. At different stages of tumor development, different tumor-targeted bacteria are administered and their effect on tumor growth and metastasis is measured over time. As the mice lack a functional immune system or have a severely compromised immune system, human tumors can be tested in this system and are not rejected by the mouse's immune system. However, as these mice are also compromised in their ability to ward off the tumor-targeted bacteria in non-tumor tissue, it may be necessary to administer antibiotics.

Pharmaceutical compositions of the invention can be tested for their ability to augment activated immune cells by contacting immune cells with a test pharmaceutical composition or a control and determining the ability of the test pharmaceutical composition to modulate (*e.g.*, increase) the biological activity of the immune cells. The ability of a test composition to modulate the biological activity of immune cells can be assessed by

detecting the expression of cytokines or antigens, detecting the proliferation of immune cells, detecting the activation of signaling molecules, detecting the effector function of immune cells, or detecting the differentiation of immune cells. Techniques well-known to those of skill in the art can be used for measuring these activities. For example, cellular proliferation can be assayed by ^{3}H-thymidine incorporation assays and trypan blue cell counts. Cytokine and antigen expression can be assayed, for example, by immunoassays including, but are not limited to, competitive and non-competitive assay systems using techniques such as western blots, immunohistochemistry, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and FACS analysis. The activation of signaling molccules can be assayed, for example, by kinase assays and electromobility shift assays (EMSAs). The effector function of T-cells can be measured, for example, by a ^{51}Cr-release assay (see, *e.g.*, Palladino *et al.*, 1987, Cancer Res. 47:5074-5079 and Blachere *et al.*, 1993, J. Immunotherapy 14:352-356).

The effect of different combinations of an immunomodulatory agent and an attenuated tumor-targeted bacteria on peripheral blood lymphocyte counts can be monitored/assessed using standard techniques known to one of skill in the art. Peripheral blood lymphocytes counts in a subject can be determined by, *e.g.*, obtaining a sample of peripheral blood from said subject, separating the lymphocytes from other components of peripheral blood such as plasma using, *e.g.*, Ficoll-Hypaque (Pharmacia) gradient centrifugation, and counting the lymphocytes using trypan blue. Peripheral blood T-cell counts in subject can be determined by, *e.g.*, separating the lymphocytes from other components of peripheral blood such as plasma using, *e.g.*, a use of Ficoll-Hypaque (Pharmacia) gradient centrifugation, labeling the T-cells with an antibody directed to a T-cell antigen such as CD3, CD4, and CD8 which is conjugated to FITC or phycoerythrin, and measuring the number of T-cells by FACS.

The expression of therapeutic molecules by tumor-targeted bacteria may be assessed. Expression of therapeutic molecules can be assayed by any technique well-known to the skilled artisan including, but not limited to, Western blot, Northern blot, RNase protection assays, enzymatic activity assays, *in situ* hybridization, immunohistochemistry, and immunocytochemistry. In any of these assays the probe to be used is specific to the therapeutic molecule whose expression is to be investigated. In one embodiment of the invention, the assay is performed on RNA preparations or on protein

preparations of tumor-targeted bacteria comprising nucleotide sequences encoding one or more therapeutic molecules. Alternatively, the assay is performed on RNA preparations or on protein preparations of solid tumor cancers treated with tumor-targeted bacteria comprising nucleotide sequences encoding one or more therapeutic molecules. In any case, negative controls of material obtained from bacteria without nucleotide sequences encoding one or more therapeutic molecules is assayed. In order to measure the relative accumulation of the therapeutic molecule in the solid tumor cancer, the ratio of strength of expression of the therapeutic molecule between the solid tumor cancer and wild-type tissue is measured.

The effect of the administration of one or more immunomodulatory agents and one or tumor-targeted bacteria to a subject with a solid tumor cancer on tissues other than the solid tumor cancer can be tested by, *e.g.*, measuring levels and activities of enzymes isolated from the liver of the mice. Such enzymes include, but are not limited to, cytochrome P450, serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic-pyruvic transaminase (SGPT). Abnormal levels and/or activities of these enzymes in the liver would indicate adverse effects of the treatment on the liver. Furthermore, the blood levels and activities of these enzymes can be measured. Increased levels and/or activities of these enzymes in the blood would indicate damage to the cells of the liver and the resulting uncontrolled release of these enzymes into the blood stream.

5.7. KITS

The invention also provides a pharmaceutical pack or kit comprising one or more containers with one or more of the components of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

In a specific embodiment of the invention, the kit comprises one or more tumor-targeted bacteria, one or more immunomodulatory agents, and optionally means of administering the pharmaceuticals of the invention. The different tumor-targeted bacteria may comprise nucleotide sequences encoding one or more therapeutic molecules. The kit may further comprises instructions for use of said immunomodulatory agents and tumor-targeted bacteria. In certain embodiments of the invention, the kit comprises a document providing instructions for the use of the composition of the invention in, *e.g.*, written and/or

electronic form. Said instructions provide information relating to, *e.g.*, dosage, method of administration, and duration of treatment.

In one embodiment, a kit of the invention comprises an immunomodulatory agent contained in a first vial, an attenuated tumor-targeted bacteria contained in a second vial, and instructions for administering the immunomodulatory agent and attenuated tumor-targeted bacteria to a subject with a solid tumor cancer, wherein the attenuated tumor-targeted bacteria is a facultative anaerobe or facultative aerobe. In another embodiment, a kit of the invention comprises an immunomodulatory agent contained in a first vial, an attenuated tumor-targeted bacteria contained in a second vial, and instructions for administering the immunomodulatory agent and attenuated tumor-targeted bacteria to a subject with a solid tumor cancer, wherein the attenuated tumor-targeted bacteria is an obligate anaerobe. In accordance with these embodiments, the tumor-targeted bacteria may be engineered to express one or more nucleic acid molecules encoding one or more therapeutic molecules. In another embodiment, a kit of the invention comprises an immunomodulatory agent contained in a first vial, an attenuated tumor-targeted *Salmonella* contained in a second vial, and instructions for administering the immunomodulatory agent and attenuated tumor-targeted *Salmonella* to a subject with a solid tumor cancer. In accordance with this embodiment, the tumor-targeted *Salmonella* may be engineered to express one or more nucleic acid molecules encoding one or more therapeutic molecules.

The kits of the invention may also comprise, means of testing the effectiveness of the pharmaceuticals of the invention. Said means of testing the effectiveness of the pharmaceuticals of the invention include, but are not limited to, tumorigenic cell lines, means of conducting a biopsy procedure, means for administering tumorigenic cells to an animal model, molecular markers (*e.g.*, antibodies and probes for *in situ* hybridization) for testing the expression of therapeutic molecules, molecular markers (*e.g.*, antibodies and probes for *in situ* hybridization) for testing the activity state of the immune system *etc.* Optionally, associated with such a kit can be a description of how to conduct said tests.

The following series of examples are presented by way of illustration and not by way of limitation of the scope of the invention.

6. EXAMPLE: THE EFFECT OF METHOTREXATE ON B16-F10 MELANOMA TARGETING BY SALMONELLA VNP20009

The following experiment demonstrates the ability of the VNP20009 *Salmonella* to target B16-F10 melanoma tumors in the presence of the immunomodulatory agent methotrexate.

B16-F10 cells grown in log phase are removed from the culture vessel by trypsination, washed in PBS, and reconstituted to about $5x10^6$ cell/ml in PBS. Single cell suspensions (0.1 ml) are injected into mice subcutaneously on Day 0 ($5x10^5$ cells/mouse) at the right flank and the animals are randomized in groups of 5 to 10 mice/group. Vials of frozen VNP20009 *Salmonella* strains are thawed quickly and diluted to about $5x10^3$ CFU/ml. Aliquots of 0.2 ml bacteria suspension ($1x10^3$ CFU/mouse) are injected intravenously into mice when tumor volumes reach about 200 mm^3. The titre of the bacteria in the injected solution can be confirmed by plating the bacteria solution on msbB plates (LB plates lacking salt). 0.2 ml of methotrexate are injected intraperitoneally at concentrations ranging from 0.5mpk to 10mpk. Methotrexate was administered to the mice on days 8, 10 and 12 when the bacteria was injected. The titre of VNP20009 bacteria in tumor tissue and liver tissue of the mice can be measured over a timecourse of several days, weeks or months after bacteria injection. Organ weights are assayed for calculation of CFU/g. Tumors and livers are homogenized and plated on msbB to determine the colony forming units (CFU) 4 days after the bacteria was injected. A dosage of 6 mpk of methotrexate administered to tumor-bearing mice significantly enhanced (one tailed Student t test, $P<0.05$) the number of VNP20009 bacteria accumulated in tumors as compared to mice that did not receive methotrexate (FIG. 1).

7. EXAMPLE: THE EFFECT OF METHOTREXATE ON CLOUDMAN MELANOMA TARGETING BY SALMONELLA VNP20009

The following experiment demonstrates the ability of the immunomodulatory agent methotrexate to augment the ability of the VNP20009 *Salmonella* to target Cloudman melanoma tumors in DBA/2 mice.

Vials of frozen VNP20009 *Salmonella* strains are thawed quickly and diluted to $5x10^3$ CFU/ml or $5x10^4$ CFU/ml. Aliquots of 0.2 ml bacterial suspension ($1x10^3$ CFU/mouse or $1x10^4$ CFU/mouse) are injected intravenously into mice when tumor volumes reached about 200 mm^3. The titre of bacteria in the injected solution can be confirmed by plating the bacteria solution on msbB plates. Methotrexate is injected intraperitoneally at doses ranging from 0.5 mpk to 10 mpk. Methotrexate can be administered repeatedly. The titre of VNP20009 bacteria in the tumor and liver of mice can be measured over a timecourse of days, weeks, or months after bacteria injection. Organ weights are assayed for calculation of CFU/g. Tumors and livers are homogenized and plated on msbB to determine the colony forming units (CFU). A dosage of 6 mpk of methotrexate administered to tumor-bearing mice significantly enhanced (one tailed

Student t test, $P<0.05$) the number of VNP20009 bacteria accumulated in tumors as compared to mice that did not receive methotrexate (FIG. 2).

8. EXAMPLE: THE EFFECT OF CYCLOSPORIN A ON B16-F10 MELANOMA TARGETING BY SALMONELLA VNP20009 IN C57BL/6 MICE

The following experiment is designed to test the ability of the VNP20009 *Salmonella* strain to target B16-F10 melanoma tumors in C57BL/6 mice in the presence of the immunomodulatory agent cyclosporin A.

C57BL/6 mice are randomized into groups of 5-10 mice. Liquid nitrogen stored B16-F10 melanoma cells are recovered by rapidly thawing the cells in a 37°C water bath. B16-F10 melanoma cells are cultured in DMEM culture medium containing 10% FBS at 37°C, 5% CO_2. After passing over two generations, B16 cells in log phase (approximately 90-95% saturation) are detached by trypsinization, washed with 1x PBS, and reconstituted to $2.5x10^6$ cells/ml with 1xPBS. Subsequently 0.2 ml of the cell suspension are injected into mice subcutaneously on Day 0 ($5x10^5$ cells/mouse) at the right flank for tumor implantation. Cyclosporin A is administered, i.p., qd for 6 days to mice on Day 11 at doses of 0.5-50mpk. Control groups are not injected with cyclosporin A. Vials of frozen VNP20009 *Salmonella* strains are thawed quickly and diluted to $1.5x10^3$ CFU/ml or $1.5x10^4$ CFU/ml. Aliquots of 0.2 ml bacteria suspension ($3x10^2$ CFU/mouse or $3x10^3$ CFU/mouse) are injected intravenously into mice on Day 12. At that time the tumor volumes should vary between 300-400mm^3. The bacterial titre of the suspension that is used to inject the mice can be confirmed by plating the bacterial suspension on msbB plates. The titre of VNP20009 bacteria in tumor and liver of mice is measured 5 days after bacteria injection. Organ weights are assayed for calculation of CFU/g. Tumors and livers are homogenized and plated on msbB at different dilutions to determine the colony forming units (CFU).

9. EXAMPLE: THE EFFECT OF CD4 AND CD8a SPECIFIC MONOCLONAL ANTIBODIES ON B16-F10 MELANOMA TARGETING BY SALMONELLA VNP20009 IN C57BL/6 MICE

The following experiment demonstrates the ability of the VNP20009 *Salmonella* strain to target B16-F10 melanoma tumors in mice in the presence of immunomodulatory antibodies specific to CD4 and CD8a.

Liquid nitrogen stored B16-F10 melanoma cells are recovered by rapidly thawing the cells in a 37°C water bath. B16-F10 melanoma cells are cultured in DMEM culture medium containing 10% FBS at 37°C, 5% CO_2. After passing over two generations, B16

cells in log phase (approximately 90-95% saturation) are detached by trypsination, washed with 1x PBS, and reconstituted to $5x10^6$ cells/ml with 1xPBS. Subsequently, 0.1 ml of the cell suspension is injected into mice subcutaneously on Day 0 ($5x10^5$ cells/mouse) at the right flank for tumor implantation. Vials of frozen VNP20009 *Salmonella* strains are thawed quickly and diluted to $5x10^3$ CFU/ml. Aliquots of 0.2 ml bacteria suspension ($1x10^3$ CFU/mouse) are injected intravenously into mice on Day 12. The bacterial titre in the suspension that is used to inject the mice can be confirmed by plating the bacterial suspension on msbB plates. Monoclonal antibodies GK1.5 (CD4/L3T4) and 53-6.7 (CD8a/Ly-2) are injected, i.p., 4 times (2 days before tumor implantation and 5, 11 and 14 days after implantation) at doses of 10-1000 μg. The titre of VNP20009 bacteria in tumor and liver of mice is measured 5 days after bacteria injection on Day 18. Organ weights are assayed for calculation of CFU/g. Tumors and livers are homogenized and plated on msbB at different dilutions to determine the colony forming units (CFU). A dosage of 500 μg/kg of anti-CD8 antibody administered every 3 to 7 days for 4 times to tumor-bearing mice significantly enhanced (one tailed Student t test $P<0.05$) the number of VNP20009 bacteria accumulated in tumors as compared to mice that did not receive the antibody (FIG. 3). Although the dosage of anti-CD4 antibody administered to tumor-bearing mice increased the number of VNP20009 in tumors, the difference was not statistically significant.

10. EXAMPLE: THE EFFECT OF CYCLOSPORIN A ON M109 LUNG CARCINOMA TARGETING BY SALMONELLA VNP20009 IN BALB/C MICE

The following experiment is designed to test the ability of the VNP20009 *Salmonella* strain to target M109 lung carcinoma tumors in mice in the presence of the immunomodulatory agent Cyclosporin A.

Balb/c mice are randomized into groups of 5-10 mice. Liquid nitrogen stored M109 lung carcinoma cells are recovered by rapidly thawing the cells in a 37°C water bath. M109 lung carcinoma cells are cultured in DMEM culture medium containing 10% FBS at 37°C, 5% CO_2. After passing over two generations, M109 lung carcinoma cells in log phase (approximately 90-95% saturation) are detached by trypsinization, washed with 1x PBS, and reconstituted to $2.5x10^6$ cells/ml with 1xPBS, and subsequently 0.2 ml of the cell suspension are injected into mice subcutaneously on Day 0 ($5x10^5$ cells/mouse) at the right flank for tumor implantation. Cyclosporin A is administered to mice at a dose of 30mpk, i.p., qd for three days starting on Day 12. Vials of frozen VNP20009 *Salmonella* strains are thawed quickly and diluted to $1.5x10^3$ CFU/ml and $1.5x10^4$ CFU/ml by standard procedures. Aliquots of 0.2 ml bacteria suspension ($1x10^3$ CFU/mouse) are injected

intravenously into mice on Day 13. At that time the tumor volumes should vary between 300-400mm^3. The titre of bacteria in the suspension that is used to inject the mice can be confirmed by plating bacteria suspension on msbB plates. The titre of VNP20009 bacteria in tumor and liver of mice is measured 5 days after bacteria injection on Day 18. Tumors and livers are homogenized and plated on msbB at different dilutions to determine the colony forming units (CFU). Organ weights are assayed the next day for calculation of CFU/g.

11. EXAMPLE: THE EFFECT OF ADMINISTRATION OF VNP20009 AND IMMUNOMODULATORY AGENTS ON TISSUES OTHER THAN THE SOLID TUMOR CANCER

The following experiment is designed to test the ability of the VNP20009 *Salmonella* strain to affect tissues other than the solid tumor cancer in mice in the presence of immunomodulatory agents.

Liquid nitrogen stored B16-F10 melanoma cells are recovered by rapidly thawing the cells in a 37°C water bath. B16-F10 melanoma cells are cultured in DMEM culture medium containing 10% FBS at 37°C, 5% CO_2. After passing over two generations, B16 cells in log phase (approximately 90-95% saturation) are detached by trypsinization, washed with 1x PBS, and reconstituted to 5x10^6 cells/ml with 1xPBS. Subsequently, 0.1 ml of the cell suspension is injected into mice subcutaneously on Day 0 (5x10^5 cells/mouse) at the right flank for tumor implantation. C57BL/6 mice are randomized into groups á 5-100 mice when the tumor volumes reach 300-500mm^3 (about Day 13). Vials of frozen VNP20009 *Salmonella* strains are thawed quickly and diluted to 5x10^3 CFU/ml. Aliquots of 0.2 ml bacteria suspension (1x10^3 CFU/mouse) are injected intravenously into mice on Day 13. At that time the tumor volumes should vary between 300-500mm^3. The bacterial titre in the suspension that is used to inject the mice can be confirmed by plating the bacterial suspension on msbB plates. Monoclonal antibodies GK1.5 (CD4/L3T4) and 53-6.7 (CD8a/Ly-2) are injected, i.p., 4 times on 3 days before and 5, 11 and 14 days after tumor-implantation at a dosage of 500 μg. Alternatively, Cyclosporin A is administered to mice at a dose of 30mpk, i.p., qd for three days starting on Day 12, or Methotrexate is injected intraperitoneally at doses ranging from 0.5mpk to 10mpk. The effect of this treatment on tissues other than the solid tumor cancer can be tested by measuring levels bacteria isolated from the liver of the mice.

12. EXAMPLE: THE EFFECT OF ADMINISTRATION OF VNP20009 AND METHOTREXATE ON THE GROWTH OF B16-F10 MELANOMA

The following experiment demonstrates the ability of an immunomodulatory agent such as methotrexate to increase the therapeutic effect of the VNP20009 *Salmonella* strain on B16-F10 melanoma.

B16-F10 cells grown in log phase were removed from the culture vessel by trypsinization, washed in PBS, and reconstituted to about $5x10^6$ cells/ml in PBS. Single cell suspensions (0.1 ml) were injected into mice subcutaneously on Day 0 ($5x10^5$ cells/mouse) at the right flank and the animals were randomized in groups of 5 to 10 mice/group. Vials of frozen VNP20009 *Salmonella* strains were thawed quickly and diluted to about $5x10^3$ CFU/ml. Aliquots of 0.2 ml bacteria suspension ($1x10^3$ CFU/mouse) were injected intravenously into mice on Day 7. Aliquots of methotrexate, at 6 mg/kg in 0.2 ml saline, were injected intraperitoneally to mice on Day 5, Day 7, and Day 9. Tumor size was measured twice weekly. FIG. 4 demonstrates that methotrexate enhances the anti-tumor effect of VNP20009 and the difference was significant (one tailed Student t test $P<0.05$).

The present invention is not to be limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All patent and non-patent publications cited herein are incorporated by reference in their entirety.

What is claimed is:

1. A method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of one or more attenuated tumor-targeted bacteria, wherein the attenuated tumor-targeted bacteria is a facultative anaerobe or facultative aerobe and the immunomodulating agent(s) is agent other than chemotherapeutic agent.

2. A method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of an anti-CD8 antibody and an effective amount of one or more attenuated tumor-targeted bacteria, wherein said attenuated tumor-targeted bacteria is a facultative anaerobe or facultative aerobe.

3. A method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of methotrexate and/or an effective amount of cyclosporin A, and an effective amount of one or more attenuated tumor-targeted bacteria, wherein said attenuated tumor-targeted bacteria is a faculative anaerobe or faculative aerobe.

4. A method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of one or more immunomodulatory agents and an effective amount of one or more tumor-targeted bacteria, wherein the tumor-targeted bacteria is an attenuated or non-pathogenic obligate anaerobe and the immunomodulatory agent is agents other than chemotherapeutic agent.

5. A method of inhibiting the growth or reducing the volume of a solid tumor cancer in a subject, said method comprising administering to said subject an effective amount of methotrexate and/or an effective amount of cyclosporin A, and an effective amount of one or more tumor-targeted bacteria, wherein the tumor-targeted bacteria is an attenuated or non-pathogenic obligate anaerobe.

6. The method of claim 1, 2, 3, 4 or 5, wherein the tumor-targeted bacteria comprises one or more therapeutic molecules.

7. The method of claim 1, 2, 3, 4 or 5 further comprising administering to said subject a bisphosphonate, an HMG CoA reductase inhibitor, an anti-angiogenic agent or radiation therapy.

8. The method of claim 6, wherein at least one of the therapeutic molecules is an anti-angiogenic factor, a cytostatic factor or a cytotoxic factor.

9. The method of claim 6, wherein at least one of the therapeutic molecules is a TNF family member.

10. The method of claim 6, wherein at least one of the therapeutic molecules is a a bacteriocin release factor (BRP).

11. The method of claim 6, wherein at least one of the therapeutic molecules is a tumor inhibitory enzyme.

12. The method of claim 6, wherein at least one of the therapeutic molecules is an anti-tumor protein, a pro-drug converting enzyme, an antisense molecule, a ribozyme, or an antigen.

13. The method of claim 4 or 5, wherein the tumor-targeting bacteria is a *Clostridium* sp. or a *Bifidobacterium* sp.

14. The method of claim 1, 2 or 3, wherein the attenuated tumor-targeting bacteria is *E. coli,* a *Salmonella sp, Yersinia enterocohtica, Listeria monocytogenies, Mycoplasma hominis* or a *Streptococcus* sp..

15. The method of claim 1, 2 or 3, wherein the attenuated tumor-targeted is a *Salmonella sp.* which comprises a genetically modified *msbB* gene, expresses an altered lipid A molecule compared to wild-type *Salmonella* sp., and induces TNF-α expression at a level less than that induced by a wild-type *Salmonella* sp.

effective amount of cyclosporin A is a dosage of 0.5 mg/kg, 1 mg/kg, 2 mg/kg, or 5 mg/kg of the subject's body weight.

27. The method of claim 1, 2, 3, 4, or 5, wherein the effective amount of the tumor-targeted bacteria is 1.0 c.f.u./kg to about 1 x 10^{10} c.f.u./kg.

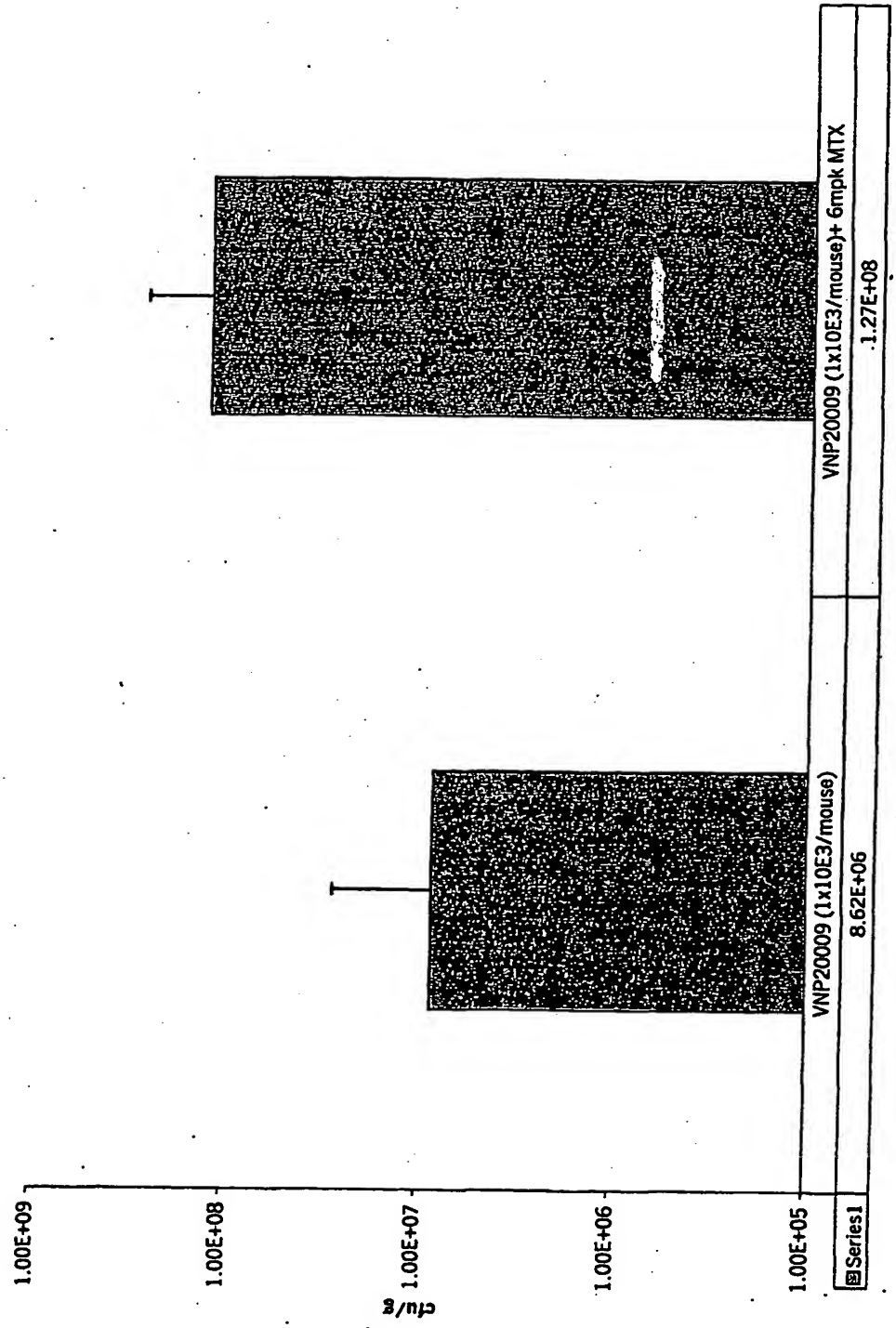
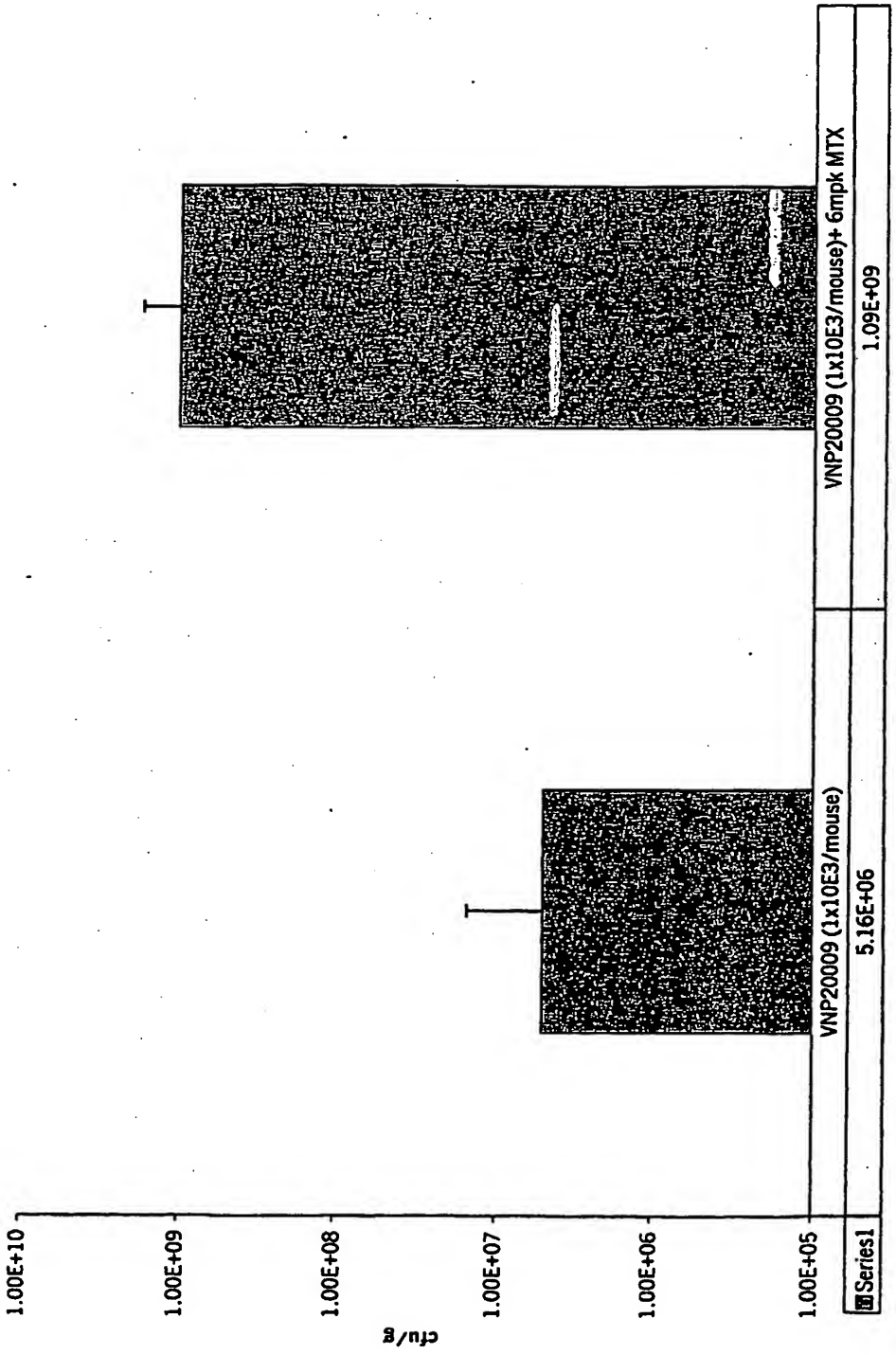

1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
cfu/g
VNP20009 (1x10E3/mouse)
8.62E+06
VNP20009 (1x10E3/mouse)+ 6mpk MTX
1.27E+08
Series1

FIG.1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1.00E+05
cfu/g
Series1
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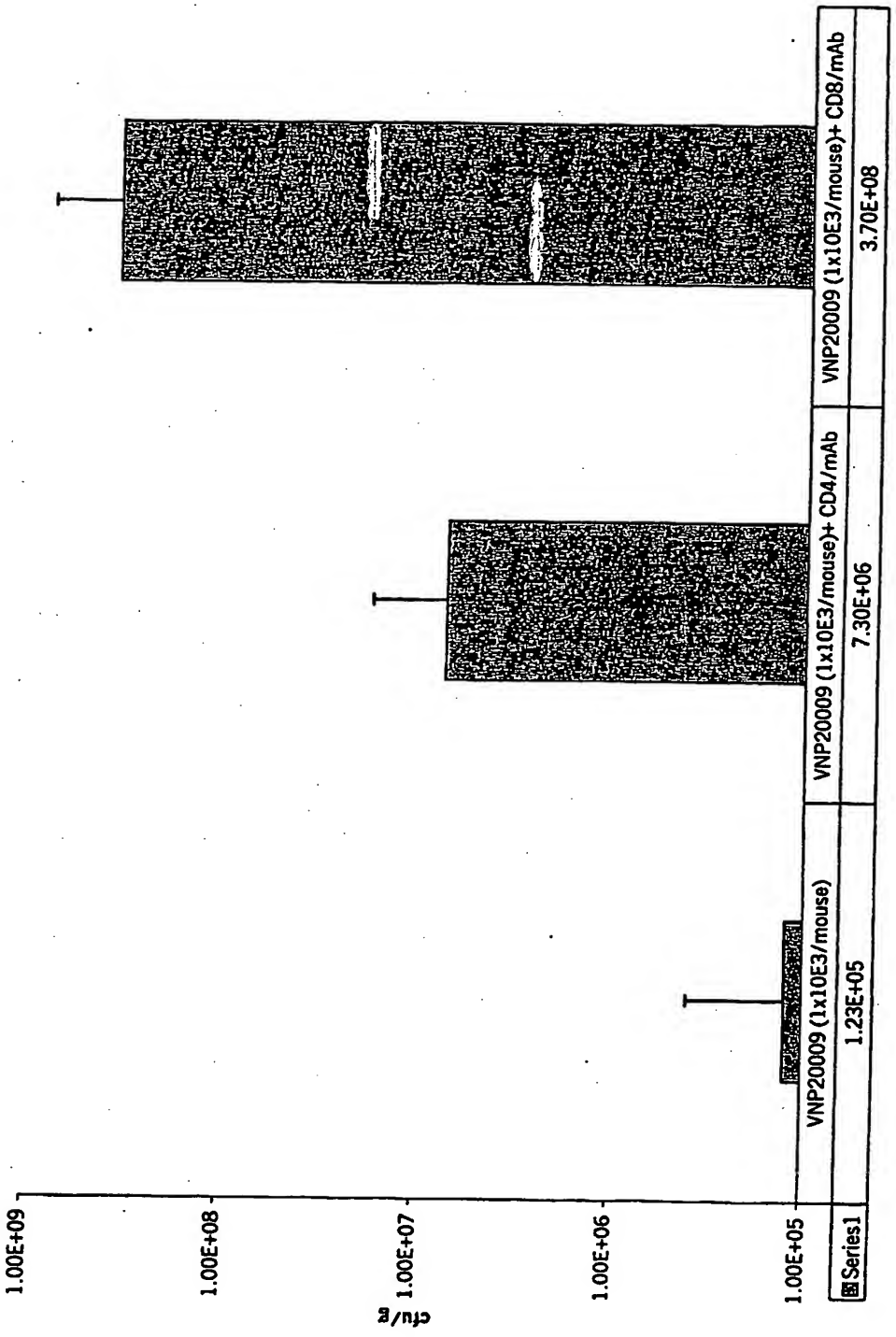
VNP20009 (1x10E3/mouse)+ 6mpk MTX
1.09E+09

FIG.2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1.00E+08
1.00E+07
1.00E+06
1.00E+05
cfu/g
Series1
VNP20009 (1x10E3/mouse)
1.23E+05
VNP20009 (1x10E3/mouse)+ CD4/mAb
7.30E+06
VNP20009 (1x10E3/mouse)+ CD8/mAb
3.70E+08

FIG.3

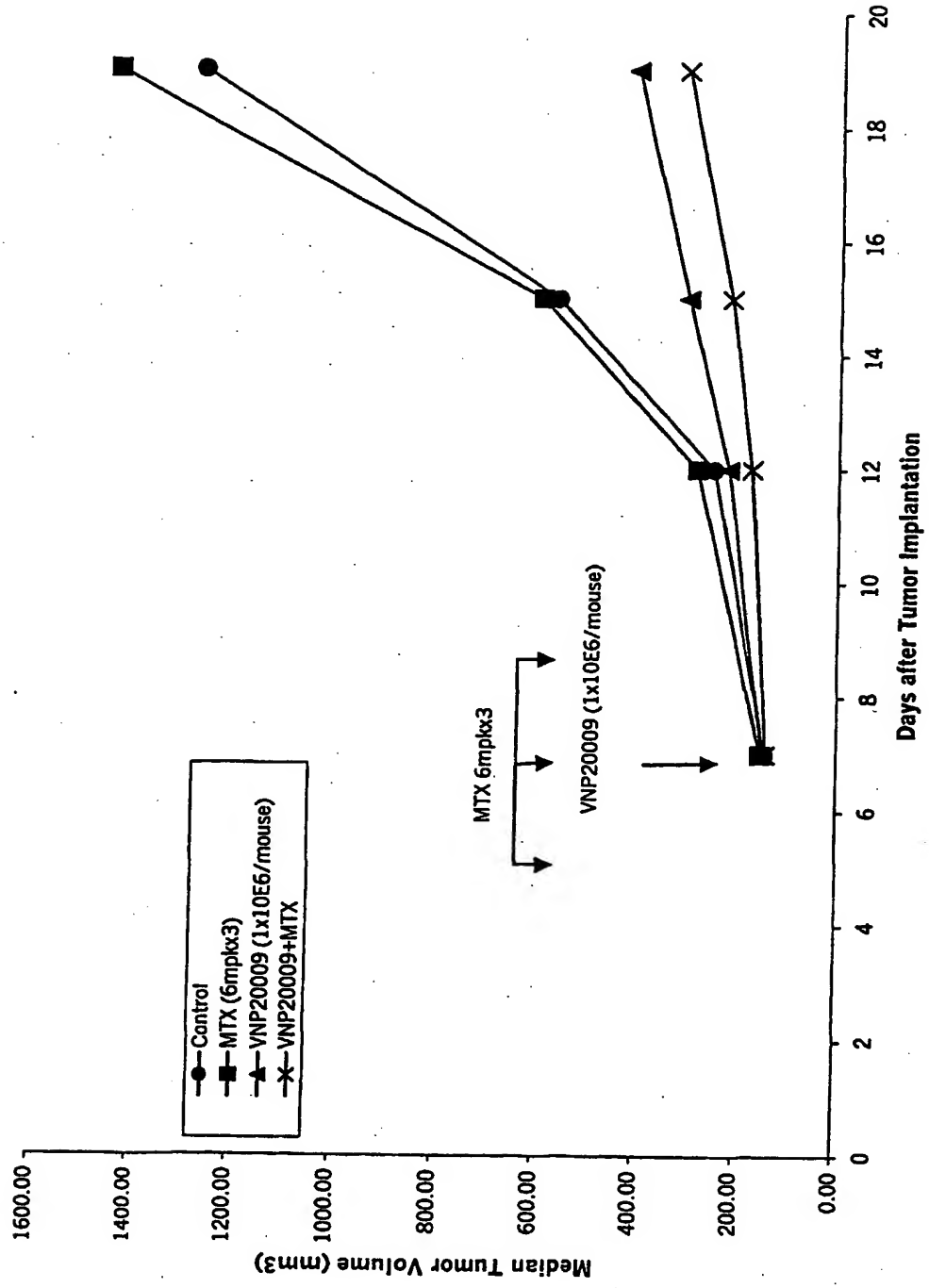
Median Tumor Volume (mm3)
1600.00
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
0
2
4
6
8
10
12
14
16
18
20
Days after Tumor Implantation
Control
MTX (6mpkx3)
VNP20009 (1x10E6/mouse)
VNP20009+MTX
MTX 6mpkx3
VNP20009 (1x10E6/mouse)

FIG.4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/02451

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01N 63/00, A61K 39/108, 39/112, 39/02, 39/09; C12N 15/00, 1/20, 15/74
US CL : 424/93.4, 93.2, 257.1, 258.1, 264.1, 244.1; 435/320.1, 252.3, 471

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/93.4, 93.2, 257.1, 258.1, 264.1, 244.1; 435/320.1, 252.3, 471

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, Y	US 6,475,482 B1 (BERMUDES et al) 05 November, 2002, see entire document.	1-27
Y	US 6,080,849 A (BERMUDES et al) 27 June 2000. see column 9-12, column 18, column 27-28.	1-27
Y	WO 01/24637 A1 (VION PHARMACEUTICALS INC.) 12 April 2001, see pages 47-51.	1-27
Y,P	WO 01/25397 A2 (VION PHARMACEUTICALS INC.) 12 April 2002. see pages 34, 40, 83, 90, 107-118.	1-27
Y	WO 99/13053 (VION PHARMACEUTICALS INC.) 18 March 1999. see entire document.	1-27
A	XIANG R. et al. An autologous oral DNA vaccine protects against murine melanoma. PNAS May 9 2000. Vol 97, No.10, pages 5492-5497, see entire document.	1-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 May 2003 (18.05.2003)	02 JUN 2003
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Sumesh Kaushal Ph.D. Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/02451

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998)